REMARKS

Prosecution

Applicants respectfully request reconsideration and withdrawal of the outstanding rejections set forth in the Office Action mailed on March 9, 2006 in view of Applicants' instant claim amendments and the following remarks. Initially, Applicants appreciate that claims 57, 63-69, 74, 79 and 81 are allowable. Several of the remaining claims have been amended to depend from an allowed claim and therefore are respectfully submitted to be in condition for allowance.

Claim Amendments

Upon entry of the foregoing amendment, claims 57, 63-69, 71, 74, 76-77, 79, 81, and 85-86 are pending in the application. Please amend claims 71 and 77 to depend from claim 57 and cancel claims 58, 80, and 83 without prejudice or disclaimer thereto. Applicants submit that the above amendments do not constitute new matter. Support for the amendments can be found throughout the specification including the claims as originally filed.

Rejection under 35 U.S.C. § 102(e)

Claims 83-84 were rejected under 35 U.S.C. § 102(e) as being anticipated by U.S. Patent 6,593,293 Baum *et al.* ("the '293 patent"). Applicants cancelled claims 83-84 rendering the rejection *moot*.

Rejection under 35 U.S.C. § 103(a)

Claims 58, 77, and 80 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over the '293 patent in view of Audtho *et al.* (October 1999); and further in view of Schenpf *et al.* (September 1998). Claims 58 and 80 have been cancelled and claim 77 now depends from claim 57 rendering this rejection *moot*.

Claims 71, 76, and 85-86 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over the '293 patent in view of Audtho *et al.*; and further in view of Schenpf *et al.*; and in further view of U.S. Patent 6,294,711 ("the '711 patent"); U.S. Patent 6,489,542 ("the 542 patent"); and U.S. Patent 6,114,608 ("the '608 patent"). Initially, the rejection with regard to 71 and 76 has

been rendered moot by the accompanying amendment. Applicants respectfully disagree and traverse this rejection with regard to claims 85 and 86.

Claim 85 is directed a chimeric gene comprising operably-linked elements including (a) a promoter region which is a DNA sequence from the Cauliflower Mosaic Virus 35S promoter; (b) a DNA encoding the TpssuAt transit peptide; (c) a DNA encoding the amino acid sequence of the protein of SEQ ID NO: 2 from an amino acid position between amino acid position 1 and amino acid position 50 to amino acid position 632; and (d) a 3' transcript termination and polyadenylation region which is a DNA sequence of the 3' transcript termination and polyadenylation region of the Cauliflower Mosaic Virus 35S gene.

In order to establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either the references themselves to modify the reference or to combine the reference disclosures. Second, there must be a reasonable expectation of success. Finally, the prior art references (or references when combined) must teach or suggest all the claim limitations. See In re Lee 277 F.3d 1338, 61 USPQ2d 1430 (Fed. Cir. 2002). With regard to element (c) the references alone or in combination fail to teach or suggest a chimeric gene comprising an isolated DNA encoding the amino acid sequence of the protein of SEQ ID NO: 2 from an amino acid position between amino acid position 1 and amino acid position 50 to amino acid position 632, *inter alia*.

The U.S.P.T.O. asserts the '293 patent discloses SEQ ID NO: 1, which can encode SEQ ID NO: 2 but "do[es] not disclose fragments of the nucleic acid encoding a protein consisting of amino acids 2-49 to 632 of SEQ ID NO:2." O. A. at 4. Additionally, the U.S.P.T.O. cites Audtho as teaching that, "another Cry2 protein, Cry2Aa1, is activated by processing at the N-terminal end, with about 49 amino acids being removed from the N-terminus." O. A. at 4. As such, the U.S.P.T.O. concludes that one of ordinary skill in the art would have found it obvious to modify the DNA sequence of the '293 patent by removing, "about 49 amino acids from the N-terminus of the protein, as described in Audtho et al." Id. The Office Action asserts that one of ordinary skill in the art would have been motivated to do so, "because truncation of the modified Cry genes results in more effective expression in plants (Schnepf et al, pg 793, left column, paragraph 3) and to avoid insect resistance (Schnepf et al, pg 795, left column, paragraph 2)." Id. at 4-5.

The '293 patent fails to disclose truncated SEQ ID NO: 2 at the N-terminus of a Cry2Ae protein as claimed. Additionally, Applicants note the '293 patent does not make a single construct, nor was any data provided that would suggest to one skilled in the art to combine this reference with the above to obtain the claimed invention. Further, Audtho is primarily concerned with the serial digestion of Cry2Aa1 to produce to fragments and not the production of a chimeric gene comprising a truncated Cry2Ae protein (SEQ ID NO: 2) as claimed. Audtho at 4602; Table 1. As such, Audtho does not teach or suggest truncating N-terminal amino acids of Cry2Ae proteins and in particular the protein of SEQ ID NO: 2. Rather, Audtho is directed Cry2Aa1 a different protein that share "rather limited homology to...other Cry proteins ...and observed to be similar to ...Cry1Aa and Cry3A." Audtho at 4601. Additionally, Audtho fails to disclose a Cry2Ae protein. Audtho compares Cry2Aa protein to the other known Cry proteins, and states that the Cry2Aa protein has "a unique mode of action" among the Cry proteins. See Id. at 4601, right col., top paragraph. Therefore, the '293 and Audtho reference fail to provide the requisite teaching or motivation to combine the references to arrive at the claimed invention.

The U.S.P.T.O. further cites to Schnepf as providing the motivation for truncation as "allow[ing] more effective expression in plants and avoids insect resistance." O.A. at 15. Schnepf, like Audtho is directed to proteins other than the claimed protein. For instance, the statement that, "truncation of the modified Cry genes results in more effective expression in plants" is directed to Cry1A proteins, not Cry2Ae as claimed. Further, passage cited by the Office Action to provide motivation "truncation allows more effective expression in plants and avoids insect resistance" cites three references:

(a) Barton *et al.* (1987) "*Bacillus thuringiensis* δ-Endotoxin Expressed in Transgene *Nicotiana tabacum* Provides Resistance to Lepidopteran Insects." Plant Physiol. **85**: 1103-1109 ("Barton") (**EXHIBIT A**);

(b) Fischhoff *et al.* (1987) "Insect Tolerant Transgenic Tomato Plants." Bio/Technology **5**: 807-813 ("Fischhoff") (**EXHIBIT B**); and

(c) Vaeck *et al.* (July 2, 1987) "Transgenic plants protected from insect attack." Nature **327**(6125): 33-37 ("Vaeck") (**EXHIBIT C**).

All three references discuss Cry1A toxins when referring to any improvement of truncation, not to any Cry2 or Bt proteins in general. Further, the alignment between the amino acid sequences of the three Cry1A-type proteins used in these three references, and the Cry2Ae protein – shows

that it is relatively small, that the maximum amino acid identity is 15% with any of the Cry1A proteins of these references. See **EXHIBIT D**. Hence, it is submitted that in the prior art the Cry2 proteins were not similar or equivalent to the Cry1A proteins of Vaeck (i.e., Cry1Ab5 in the alignment), Fischhoff (Cry1Ab10 in the alignment) and Barton (Cry1Aa1 in the alignment) (note that the names used in Crickmore et al. '98 (cited previously) are used). See **EXHIBIT D**. From this alone, it is clear that Schnepf cannot and does not address using or modifying the Cry2Ae protein. As such, one would not be motivated to combined Schnepf with Audtho and the '193 patent to obtain a chimeric gene comprising truncated Cry2Ae.

Further the U.S.P.T.O. suggests that Schnepf provides the motivation to truncate the Cry2Ae gene to "avoid insect resistance." However, the truncated Cry1A proteins in Schnepf resulted in comparatively low expression and therefore would not suggest to one skilled in the art to a motivation to combine Schnepf with the teachings of the '293 patent and Audtho. Rather, Schnepf's focuses on the "proposed strategies" to avoid insect resistance development include the use of multiple toxins, crop rotation, high or ultrahigh dosages, and spatial or temporal refugia, not truncation of Cry2Ae. Schenpf at 796. As such, the '293 patent, Audtho and Schnepf alone and in combination fail to teach the limitation of claim 85, an in particular with respect to element (c).

Similar to the above references the '711 patent is silent on Cry2Ae and does not relate to Cry2Ae expression in plants. The '711 patent reference relates to production of Cry protein in plants using different promoters independent from RNA polymerase II. A person of ordinary skill in the art at the time the application was filed would not consult the '711 patent for a chimeric gene driven by a 35S promoter, which is dependent on RNA polymerase II. See '711 patent at Col. 4-5. In particular, this reference is directed to a different field and a different expression systems and therefore does not provide the motivation to combine the above cited references to obtain the claimed invention.

Similarly, the '542 patent fails to disclose a Cry2Ae and lacks the teaching to combine the five references to obtain the claimed invention. Indeed, adding a transit peptide to a Cry2Aa gene for expression in plants resulted in abnormal plant phenotypes and significantly decreased expression in plants as discussed in the '542 patent. As such, one of ordinary skill in the art would not be motivated to add a transit peptide to Cry2Ae as claimed, particularly in view of the poor results obtained in the '542 patent. Col. 50, ll. 64 - Col. 51. ll. 4.

The '608 patent does not teach Cry2Ae nor does it relate to Cry2Ae expression in plants. The '608 patent is primarily concerned with the expression of a specific Cry1Ab gene. Therefore the '608 patent does not teach or suggest any of the limitations of claim 85, nor does the '608 patent provide any motivation or reasonable expectation of success.

For at least these reasons, the Office Action fails to establish why or how it would have been obvious to one of ordinary skill in the art at the time of the invention to combine the disclosures of the cited references to produce the chimeric gene meeting all the elements of claims 85-86. Nor does the Office Action establish motivation to combine references or demonstrate a reasonable expectation of success in combining the references to produce the claimed invention.

Again, the statutory standard of 35 U.S.C. § 103 is whether the invention, considered as a whole, would have been obvious to one of ordinary skill in the art, not whether it would have been obvious for one of ordinary skill in the art to try various combinations. Akzo N.V. v. E.I. duPont de Nemours, 1 U.S.P.Q.2d 1705, 1707 (Fed. Cir. 1987). In the instant case, the U.S.P.T.O. has selected disparate portions of references directed to gene other than Cry2Ae. However, one cannot pick and choose among individual parts of assorted references as a mosaic to recreate a facsimile of the claimed invention. Akzo N.V. v. International Trade Commission, 1 U.S.P.Q.2d 1241, 1246 (Fed. Cir. 1986). Indeed, each of the cited references fails to provide the motivation to combine their disclosures to achieve the claimed invention.

Finally, assuming *arguendo*, it were proper to combine the references, it has not been established in the Office Action that the combination would have yielded the chimeric gene of claim 85. Further, Applicants submit that dependent claim 86 is not obvious for the same reasons discussed *supra* for claim 85.

Reconsideration and withdrawal of the rejection is respectfully requested.

CONCLUSION

Applicants respectfully submits that claims 57, 63-69, 71, 74, 76-77, 79, 81, and 85-86 are in condition for allowance, and such disposition is earnestly solicited. Should the Examiner believe that any patentability issues remain after consideration of this Response, the Examiner is invited to contact the Applicants' undersigned representative to discuss and resolve such issues.

Respectfully submitted,

HUNTON & WILLIAMS LLP

Dated: September 11, 2006

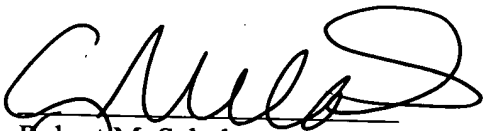
By: 

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EXHIBIT A

Barton *et al.* (1987)

"*Bacillus thuringiensis* δ-Endotoxin Expressed in Transgene *Nicotiana tabacum* Provides Resistance to Lepidopteran Insects." Plant Physiol. **85**: 1103-1109

0032-0889/87/85/1103/07/$01.00/0

Bacillus thuringiensis δ-Endotoxin Expressed in Transgenic *Nicotiana tabacum* Provides Resistance to Lepidopteran Insects

Received for publication August 26, 1987 and in revised form September 9, 1987

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ABSTRACT

The crystal proteins, or δ-endotoxins, of *Bacillus thuringiensis* are specifically lethal to Lepidopteran insects. We utilized a truncated and modified portion of a cloned crystal protein gene to construct a chimeric gene capable of expression in plant cells. Using an *Agrobacterium tumefaciens* binary vector system, we then transferred the chimeric toxin gene into tobacco (*Nicotiana tabacum* cv Havana 425) cells and regenerated recombinant plants. One to several copies per cell of the toxin gene were routinely present in the recombinant plants. Hybridization experiments demonstrated that these plants had a new RNA species of the size expected for the truncated toxin mRNA, and a polypeptide having the mobility expected for the truncated toxin was detected by immunoblotting. Significant variation was found in the levels of toxin-specific RNA expression between different recombinants, but the levels of hybridizing RNA in transformants correlated with the level of toxicity demonstrated against *Manduca sexta* (tobacco hornworm), and other Lepidopteran insects. The recombinant genes were transmitted to progeny and resistance to insects was maintained, thus demonstrating that the introduction of toxin genes into plants may be a practical method of providing protection against certain insect pests.

Bacillus thuringiensis is a bacterium that forms insecticidal proteinaceous crystals during sporulation (2, 21, 28). The crystal protein, representing as much as 30% of the spore dry weight, consists predominately of one or more protoxin species of up to 140,000 D. The protoxins can be cleaved by proteolysis to polypeptides of 55,000 to 70,000 D that are specifically toxic to Lepidopteran, Dipteran, or Coleopteran insects (2, 21, 28). Deletion analyses have localized the toxic portion of the protoxins to the amino-termini and have shown that both amino- and carboxyl-terminal fusions can be made to the toxin without loss of insecticidal activity (23). While the precise mode of action of the toxin is not yet known, ingestion of toxin by susceptible insects results first in a paralysis of the gut and mouth parts, followed by a reduction or cessation of feeding, a gradual deterioration of the gut epithelial cells, and finally death of the organism (2).

Microbial formulations of *B.t.* toxins[1] have been applied to an increasing variety of agricultural crops for over 20 years, with current worldwide annual use in excess of 2.3 million kg (21). Practical limitations such as poor persistence (25) and the relatively high costs incurred by multiple applications have limited product acceptance. However, major insect pests are developing resistance to most classes of chemical insecticides, and environmental damage caused by insecticidal chemicals is an increasing public concern. The high specificity of the *B.t.* toxins make them an attractive alternative to petrochemicals if efficiency of use can be improved. For this reason, we and others (10, 27) have explored the feasibility of generating Lepidopteran-resistant transgenic plants. This report presents data on the development of transgenic tobacco plants that produce sufficient levels of *B.t.* toxin to either kill Lepidopteran insects or inhibit their feeding. Analysis of the expression of the chimeric gene in the resistant plants has revealed several problems which affect the levels of toxin gene expression. These problems must now be resolved in order to obtain additional insect resistant agricultural crops.

MATERIALS AND METHODS

Source of Materials. Seeds of tobacco (*Nicotiana tabacum* cv Havana 425) were surface sterilized (26) and germinated on Murashige and Skoog medium (18); aseptically grown leaves and stems were used for transformations, described below. *Agrobacterium tumefaciens* strain EHA101 (15) was obtained from E. Hood. *A. tumefaciens* binary vector components have been described previously (26). DNA encoding a δ-endotoxin from *B. thuringiensis* var kurstaki HD-1-Dipel (specific for Lepidopteran insects) was obtained from plasmid pES1 (24). Eggs of *Manduca sexta* (tobacco hornworm) were purchased from Carolina Biological Supply. Eggs of *Heliothis virescens* (cotton bollworm), *Heliothis zea* (corn earworm), and *Spodoptera exigua* (beet armyworm) were purchased from the USDA Southern Regional Research Laboratory. Enzymes utilized in recombinant manipulations were purchased from New England Biolabs (Beverly, MA) and used as directed, unless otherwise noted.

pAMVBTS Construction. The plasmid AMVBTS consists of the ampicillin-resistant vector pCMC66 (26) containing a chimeric truncated *B.t.* toxin gene. The complexity of the chimeric gene required that various intermediate plasmids be constructed prior to completion of pAMVBTS, with the various components then ligated together in the final plasmid. The transcriptional promoter on the chimeric *B.t.* gene corresponds to nucleotides 7013 to 7440 of the CaMV sequence (12), which comprises the 35S promoter and enhancer region (19). This fragment was excised from pCaMV10 (14), with the ends of the fragment modified by addition of commercial linkers. The 5′ end of the promoter fragment, originally a blunt *Hin*cII restriction site, was converted to *Xho*I by ligation directly to commercial linkers. The 3′ end of the promoter fragment, originally an *Hph*I site, was first blunted by reaction with Klenow polymerase, then ligated to commercial *Hin*dIII linkers. The sequence of the region near the new *Hin*dIII site is displayed in Figure 1, with the *Hin*dIII site located at nucleotide +4 relative to the predicted start of transcription (19). The 5′ untranslated region of the gene in pAMVBTS (Fig. 1) was generated by synthesis of two comple-

[1] Abbreviations: *B.t.* toxin, *Bacillus thuringiensis* δ-endotoxin; CaMV, cauliflower mosaic virus; AMV, alfalfa mosaic virus; NPT-II, neomycin phosphotransferase-II; kb, kilobase pairs.

mentary oligonucleotides that anneal to form compatible *Hin*dIII and *Nco*I sticky ends, with the internal sequence corresponding to the untranslated region of AMV RNA 4 (13). The *Hin*dIII end of the oligonucleotide was ligated to the CaMV promoter fragment, and the *Nco*I end was ligated to the 5′ terminus of the *B.t.* toxin coding sequence.

The truncated *B.t.* toxin coding sequence on pAMVBTS corresponds to nucleotides 527 to 2463 of the sequence (24). The 5′ terminus of the coding region present on pSYC823 (a pUC8 vector containing the subcloned *B.t.* toxin gene found on pES1), was converted to an *Nco*I restriction site, CCATGG, with the use of a polymerase chain reaction and synthetic primers (22). The internal 'ATG' of the *Nco*I site represents the initiation codon of the toxin. The 3′ terminus of the truncated toxin was generated by first cleaving the toxin gene with *Bcl*I at nucleotide 2458 (24), followed by ligation of a synthetic linker to the *Bcl*I site. The sequence of the linker, formed by annealing two homologous oligonucleotides that generated *Bcl*I and *Pst*I ends, is noted in Figure 1. The linker added two additional proline codons to the toxin, then terminated the peptide with two adjacent termination codons. The *Pst*I end of the termination linker was ligated to a DNA fragment encoding the polyadenylation region of nopaline synthase as described previously (26).

Construction of Agrobacterium Vector pTV4AMVBTS. The components of the *A. tumefaciens* binary vector system used in these studies were similar to those described by Umbeck *et al.* (26), except that pTV4 was substituted for pCMC92 as the initial carrier plasmid and *A. tumefaciens* strain EHA101 (15) served as the transfer host. The vector pTV4 (Fig. 1) is a derivative of pCMC92 (26) in which the region of Ti plasmid DNA present on pCMC92 5′ to the nopaline synthase promoter has been deleted, and replaced on pTV4 with synthetic DNA consisting of the Ti plasmid right border of pTiT37 (29) and a consensus overdrive (20) derived from plasmids pTiT37 (9) and pTiA6NC (20).

Plasmid AMVBTS was co-integrated into pTV4 by ligation at unique *Xho*I restriction sites on each plasmid. The orientation as shown (Fig. 1) places the CaMV 35S enhancer (19) on the pAMVBTS 35S promoter adjacent to the nopaline synthase promoter, and thus provides a bidirectional enhancement. The plasmid TV4AMVBTS was conjugated into *A. tumefaciens* strain EHA101 (15) as previously described (3).

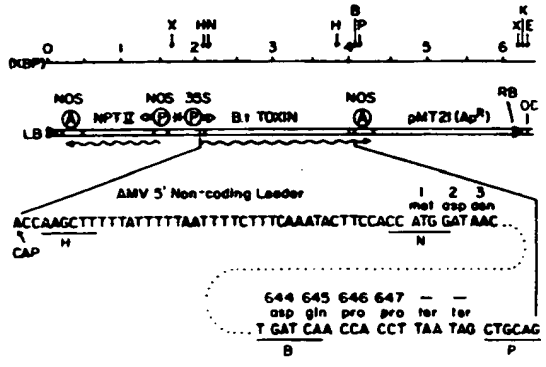

FIG. 1. Structure of pTV4AMVBTS transfer region. Plasmid TV4AMVBTS is a 16 kb wide-host-range component of an *A. tumefaciens* binary vector system (26), formed by co-integration at unique *Xho*I sites (shown) of 4.6 kb pAMVBTS and 11.6 kb pTV4. Displayed is the T-DNA region with left (LB) and right (RB) T-DNA borders of pTiT37 (29) bracketing chimeric genes encoding a selectable marker (NPT-II) and a truncated *B.t.* toxin. Scale (top line) is kilobase pairs (KBP). Abbreviations: Nos A and Nos P, nopaline synthase polyadenylation region and promoter, respectively (9); 35S P, CaMV 35S promoter (19); pMT21(ApR), ampicillin resistant plasmid pMT21; OD, consensus overdrive (20); AMV 5′noncoding leader refers to untranslated region of AMV RNA 4 (13); toxin amino- and carboxy-terminal codons are noted and numbered; * is the 35S enhancer. Restriction enzymes (underlined): *Bcl*I (B), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Nco*I (N), *Pst*I (P); *Xho*I (X).

Plant Transformations. Aseptically grown immature stems and leaves were inoculated with overnight cultures of *A. tumefaciens* (26). Following 48 to 72 h of incubation at room temperature on regeneration medium (MS medium [18] plus 1 mg/ml kinetin), cefotaxime (100 μg/ml) and vancomycin (250 μg/ml) were applied to kill the agrobacteria, and kanamycin (100 μg/ml) was applied to select transformed plant tissues. After approximately 6 weeks with media changes at 2-week intervals, shoots appeared. Shoots were excised and placed on rooting medium (3) plus 25 mg/L kanamycin until roots had formed (1–3 weeks), after which time plants were transferred to commercial soil mixture (Metro-Mix 360, W. R. Grace & Co.). Approximately 2 weeks after potting, insect toxicity tests were initiated.

Insect Toxicity Assays. Insect eggs were hatched on mature wild-type tobacco plants, and larvae were allowed to graze for 1 to 3 d prior to transfer to test plants. Mature tobacco plants contain higher levels of secondary metabolites than freshly regenerated plants, so larvae with feeding exposure to older plants were considerably less sensitive to *B.t.* toxin than neonatal larvae. The decreased sensitivity proved useful in distinguishing between variations in toxin levels in transgenic plants. Tobacco hornworms were placed directly on leaves of young wild-type and recombinant plants, usually two to four larvae per plant per test with up to six successive tests conducted per plant. Only test plants showing 100% toxicity in all tests were considered to be resistant. Alternatively, tests were conducted using excised leaf tissue in Petri dishes, with 5 to 10 hornworms or a single larva of other species per dish. In dish assays, weights of larvae were recorded at inititation and termination of tests. Feeding trials were generally 2 to 4 d in duration, with daily monitoring of reductions in feeding and larval deaths.

Southern Blot Analysis. Total nucleic acid was prepared from young plant tissues as described by Dellaporta (8). Ten μg DNA, estimated by agarose gel staining and fluorimetry, was digested with appropriate restriction enzymes and samples were then electrophoresed on 0.6% agarose. DNA was transferred to nylon membranes (Biodyne membranes, Pall), and hybridizations were conducted using [^{32}P]RNA probes as previously described (26). Filters were autoradiographed on X-AR5 film at −80°C with two intensifying screens (Cronex Lightning Plus, DuPont).

RNA Hybridizations. RNA standard transcripts for purposes of size and concentration comparison were synthesized using Riboprobe transcription kits (Promega Biotec). Templates were either NPT-II or truncated *B.t.* toxin coding regions of defined length and [^{3}H]CTP (Amersham) was incorporated at known specific activity for accurate concentration determination. Total RNA was isolated from plant leaves as described by Chirgwin *et al.* (7), except that the concentration of guanidinium thiocyanate was increased to 5 M. Following resuspension of RNA pellets after CsCl centrifugation, 10 to 40 μg RNA were either prepared for slot-blot hybridizations as recommended by the supplier of the apparatus (Schleicher & Schuell) or electrophoresed on formaldehyde-containing agarose gels as described by John *et al.* (16). After electrophoresis, gels were blotted onto nitrocellulose for hybridizations (16). [α-^{32}P]dCTP-DNA oligonucleotides were synthesized as probes for hybridizations as described for the Oligolabeling Kit (Pharmacia). Hybridizations were conducted overnight at 42°C in buffer (50 mM Tris-HCl, 3 mM Na-pyrophosphate, 1% SDS, 5 mM EDTA [pH 7.5]) containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), 150 μg/ml salmon sperm DNA, and 10× Denhardts' solution (0.2% BSA, 0.2% Ficoll, 0.2% PVP). Filters were extensively washed, with a final wash in 0.1× SSC at 60°C. Hybridization signals were detected by autoradiography for 1 to 3 d at

Table I. *Toxicity in Regenerated AMVBTS Tobacco Plants*

Kanamycin-resistant plants were regenerated following transformation with pTV4AMVBTS. The number of inserted copies of AMVBTS genes were determined by restriction mapping and Southern hybridizations, the level of toxin-related RNA (pg/20 μg) in each plant was determined (see Fig. 4A and "Materials and Methods"), and toxicity of plants to *M. sexta* larvae was evaluated. Little or no toxicity is scored (−); (+) to (++++) refer to increasing reductions in feeding leading to complete larval mortality. The ratio in 'Toxicity' refers to (no. larvae killed/no. tested); H425 refers to wild-type tobacco.

Plant No.	Genes	RNA	Toxicity
H425	0	ND[a]	−(0/50)
857	3	47	++++(12/12)
858	0	1.2	−(1/6)
859	2	1.1	+++(10/10)
860	1	0.8	+++(8/8)
861	2	1.4	++(8/8)
862	5	7	++++(8/8)
863	1	0.5	+(6/6)
870	3	2.5	+++(10/10)
872	3	1.3	++(8/8)
884	0	2.8	−(2/6)

[a] ND, Undetectable.

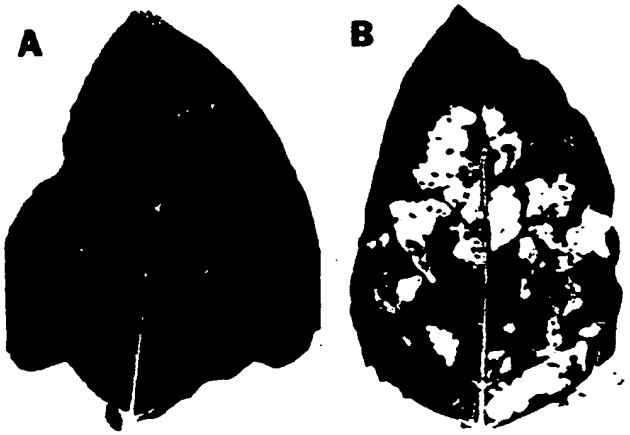

FIG. 2. Bioassay of AMVBTS transformant No. 857. Leaves of plant No. 1274 (A) and untransformed H425 (B) were placed in 10 cm Petri dishes, and five larvae of *Manduca sexta* were applied. Incubation was carried out at 25° C in the dark for 48 h, with monitoring of feeding reduction and mortality recorded at 24 and 48 h. All five larvae feeding on plant No. 1274 were dead at 24 h; the larvae on H425 leaves continued to feed until termination of the test.

−80°C using X-AR5 film with two intensifying screens (Cronex Lightning Plus, DuPont).

Protein Extraction and Immunoblots. Production of truncated *B.t.* toxin for use in insect feeding trials and as standards on immunoblots was accomplished by substituting the *tac* promoter on pKK233-2 (1) for the CaMV 35S promoter on pAMVBTS, and growing the host *E. coli* under conditions which provide for *tac* promoter induction (1). For extraction of toxin from recombinant plants, young leaf tissue was frozen in liquid N_2, powdered, then sonicated briefly in an equivalent (w/v) amount of extraction buffer (50 mM Tris-HCl, 100 mM NaCl, 0.5% NP40, 1% 2-mercaptoethanol, 4% SDS, 2.5 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, and 50 μg/ml each of antipain, pepstatin, and aprotinin, final pH 12.5). Immediately following sonication, extracts were boiled and supernatants were collected following microcentrifugation. Acrylamide gel electrophoresis of supernatants and toxin standards was conducted, proteins were blotted onto nitrocellulose, and enzyme-linked immunochemical staining was carried out (5, 6). Briefly, nitrocellulose blots were incubated with rabbit anti-*B.t.* toxin serum (1:1000 dilution) and subsequently with alkaline phosphatase-conjugated anti-rabbit immunoglobulin (1:1000 dilution). The immunoblots were stained with 5-bromo-4-chloro-3-indoylyl phosphate (*p*-toluidine salt, Sigma) and *p*-nitro-blue-tetrazolium chloride (Sigma).

RESULTS

***B.t.* Toxin Gene and Vectors.** Isolation and sequence analysis of the δ-endotoxin gene of *B. thuringiensis* var kurstaki HD-1-Dipel has been described (24). Of the 1176 amino acids present in the crystal protein only approximately 600 amino-terminal residues are required for insect toxin activity (23). The function of the remaining carboxy portion of the protoxin, beyond structural considerations in crystal formation, remains unknown. Because the intact protoxin had seen extensive field use in microbial formulations, we initially attempted to express the entire 1176 amino acid toxin in plant cells. Our initial constructions were similar to those detailed here, but contained the entire toxin coding sequence. Following transformation and selections for kanamycin resistant transformants, we obtained tobacco calli that were shown by immunoblots to contain significant levels of intact protoxin (10–50 ng/mg protein). However, all such calli soon became necrotic and died. Any plants we were able to regenerate from our initial experiments were shown by hybridization analysis and immunoblots to contain either broken or inactive toxin genes (data not shown). We eventually concluded that expression of intact δ-endotoxin was lethal to plant cells. In subsequent experiments, including those described in this report, we eliminated the protoxin carboxy terminus and found no evidence that the truncated toxin is deleterious to plant cell viability.

The plasmid AMVBTS is an expression cassette encoding the truncated *B.t.* toxin, under the transcriptional control of the CaMV 35S promoter (Figure 1). Adaptation of the promoter fragment for use in chimeric gene construction deleted the entire 35S mRNA coding region, with the exception of the 5′ cap site and the penultimate nucleotide (19). We, therefore, reconstructed an mRNA 5′ untranslated region for the toxin transcript by synthesizing a synthetic duplex oligonucleotide corresponding in sequence to the 5′ end of the AMV RNA 4 (13). The AMV RNA 4, which encodes the coat protein, is translated efficiently both *in vivo* and *in vitro*, potentially because of characteristics found in the untranslated region of the RNA (13).

The *B.t.* toxin coding region was modified as described in "Materials and Methods" to have an *Nco*I restriction site at the amino terminus, with nucleotides neighboring the first 'ATG' codon chosen to conform to Kozak's consensus for translational initiation in eucaryotes (17). The toxin was truncated after codon 645, which is 3′ to the proteolytic cleavage site that generates the mature toxin in the insect gut (23). The synthetic linker we ligated to the terminus of the truncated toxin gene supplied two adjacent proline codons followed by two termination codons. The prolines, which are both hydrophobic and resistant to various proteases (4), might be expected to help protect the new carboxy terminus of the peptide.

The binary Ti plasmid vector system used in this set of experiments is similar to that previously reported (26). However, modifications have been introduced in the 'carrier plasmid' TV4, the T-DNA border portion of the vector system. The replacement of an authentic Ti plasmid right border region from pTiT37, as found on pCMC92 (26), with the synthetic T-DNA border and overdrive sequence described here is useful because it decreases the plasmid size by approximately 1.5 kb (to 12 kb) and adds convenient unique restriction sites (including *Xho*I) between our selectable marker (NPT-II) and the border region. Placement of the gene of interest in the *Xho*I site between the marker and the

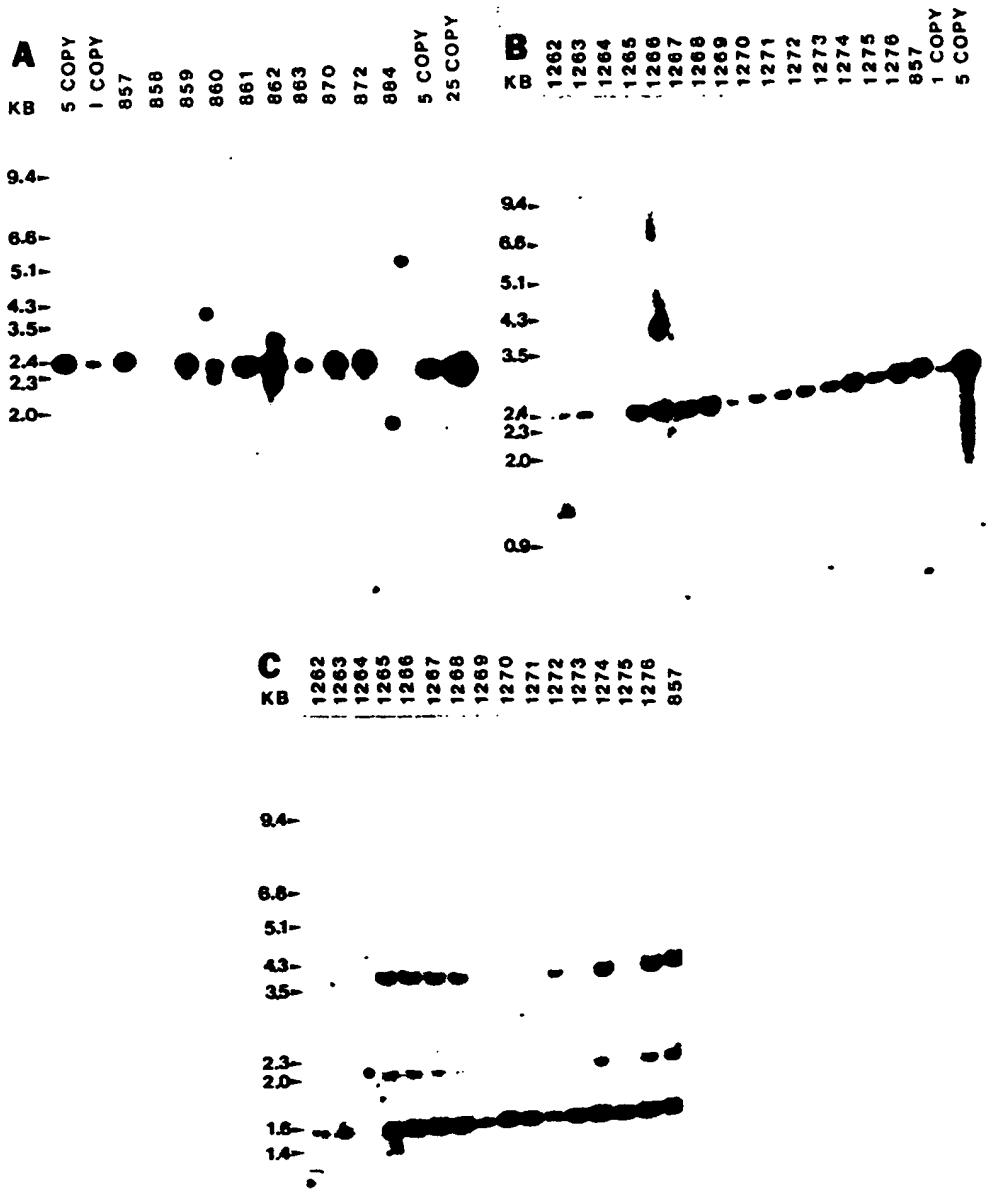

FIG. 3. Southern blot analysis of AMVBTS insertions in regenerated tobacco plants and progeny. Plant DNA (10 μg) was digested with both endonucleases *Xho*I and *Pst*I (A and B) or *Xho*I (C) and electrophoresed on 0.6% agarose gels. DNA was then transferred onto nylon membranes, and hybridized to [^{32}P] RNA probes homologous to the *B.t.* toxin (A and B) or NPT-II (C). DNA copy standards are pTV4AMVBTS plasmid at the calculated concentrations for 1, 5, or 25 insertions per cell combined with 10 μg tobacco DNA. Mobility of standard DNA fragments is noted (KB). A, DNA from regenerated AMVBTS tobacco plants; B and C, DNA from progeny of plant No. 857.

.t border generates fewer plant transformants with broken or deleted target genes, since the right border is relatively precise in transfer to plants (29). Use of these synthetic sequences on pTV4 provides no apparent differences in transformation efficiency from our previous plasmids, such as pCMC92 (26).

Plasmid AMVBTS was cointegrated into pTV4 using unique *Xho*I restriction sites on each plasmid. The orientation as shown (Fig. 1) places the CaMV 35S promoter and associated enhancer (19) adjacent to the nopaline synthase promoter (9), and thus is likely to provide a bidirectional enhancement affecting both the NPT-II and toxin genes. This orientation of the two co-integrated plasmids further provides for greater plasmid stability than the opposite orientation, which would result in direct repeats of the nopaline synthase polyadenylation region on the two genes.

Transformation of tobacco tissues with our *A. tumefaciens* vectors resulted in kanamycin-resistant shoots in approximately 6 weeks. The majority of the excised shoots readily formed roots when placed in kanamycin-containing medium. At that time, the young plants were transferred to soil, and growth was continued until completion of insect toxicity tests.

Insect Toxicity. Table I shows the toxicity analysis of one set of 10 AMVBTS transformants. Relative levels of toxicity between those plants providing complete *M. sexta* larvae mortality are subjective, and are based on the extent of damage to plant leaves prior to cessation of feeding. In all cases of mortality, some feeding on test tissues was observed (Fig. 2). Analysis of over 100 independent transformants has shown that approximately 25% of the plants were lethal to all larvae within 4 d, with the more resistant plants allowing only minimal feeding during the early hours of the test. Many plants judged 'nontoxic' (few larvae were killed) did reduce larval feeding levels and growth rates in comparison to control tissues.

Southern Blots. The results of Southern blot analyses on the 10 regenerated AMVBTS plants of Table I are shown in Figure 3A. Digestion of the plant DNA with *Pst*I and *Xho*I should release the toxin chimera as a 2.42 kb internal DNA fragment, which includes the CaMV promoter and the entire toxin coding region (see Fig. 1). Eight of the 10 plants apparently had one or more intact toxin genes, while plants No. 858 and No. 884 showed only broken inserts (hybridizing fragments in plant No.

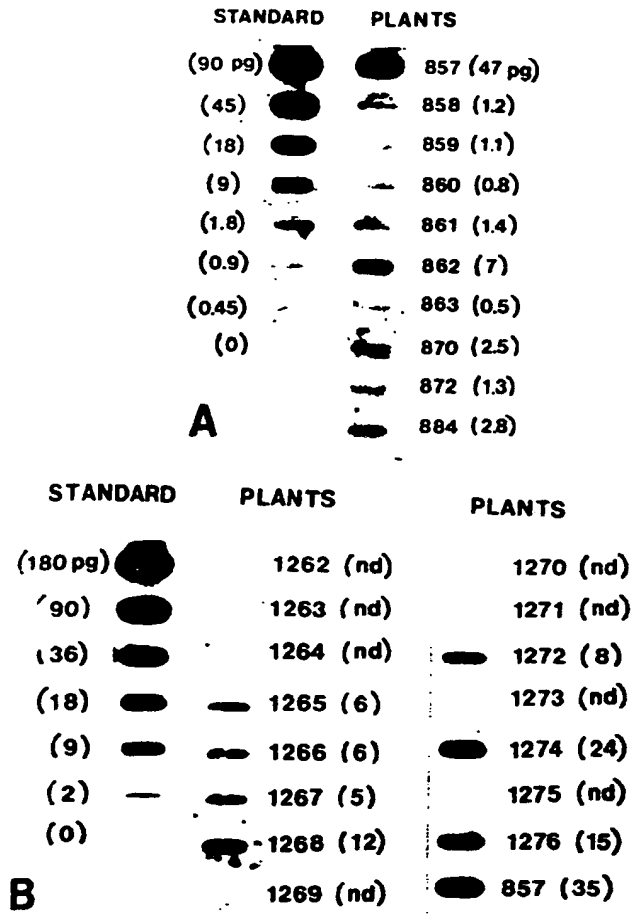

FIG. 4. RNA slot-blot analysis of regenerated plants and progeny. Total RNA (20 μg) isolated from regenerated AMVBTS plants (A) or progeny of plant No. 857 (B) were analyzed as described in "Materials and Methods." Standard toxin RNAs were synthesized as [^{3}H]RNA of defined specific activity for accurate quantification, and combined with 20 μg tobacco RNA prior to binding to filters. Labeled [^{32}P]DNA probes homologous to *B.t.* toxin were hybridized to the filters, which were visualized by autoradiography. Quantification (pg) of test plants is by densitometry on autoradiograms relative to standard transcripts; nd is "not detectable."

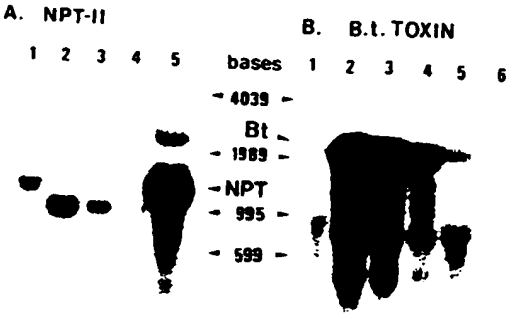

FIG. 5. Northern blot analysis of NPT-II and *B.t.* toxin gene transcripts in plant No. 1265. RNA isolated from plant No. 1265 was electrophoresed on formaldehyde formamide-containing agarose gels, transferred onto nitrocellulose and probed with [^{32}P]DNA homologous to NPT-II (A) or *B.t.* toxin (B) genes. Standard [^{3}H]RNA transcripts for NPT-II and *B.t.* toxin were used for quantification of plant RNAs; *in vitro* synthesized RNA transcripts of defined length were used as size markers (center). A, Lane 1, 20 μg total RNA of No. 1265; lanes 2 to 4, 20 μg H425 RNA plus 100, 50, and 10 pg *in vitro* synthesized NPT-II transcripts, respectively; lane 5, 17 μg oligo-dT selected RNA of No. 1265; B, lane 1, 20 μg total RNA of No. 1265; lanes 2 to 4, 20 μg H425 RNA plus 180, 90, and 35 pg *in vitro* synthesized *B.t.* toxin transcripts; lanes 5 and 6, 28 μg and 17 μg oligo-dT selected RNA of No. 1265 and H425, respectively. Locations of apparent *B.t.* toxin (Bt) and NPT-II (NPT) transcripts are noted.

85[illegible] of variable sizes, all less than single copy, and not visible in Fig. 1). Additional digests to analyze the border fragments with recombinant genes attached to plant DNA (3) indicated that each transformant with intact genes contained between one and three different inserts that hybridized at single-copy intensity (data not shown). The relative proportion of intact inserts, copy numbers, and overall frequency of regeneration in these and our other AMVBTS plants compare favorably to our experience with other genes, thereby supporting the idea that this truncated toxin does not have the deleterious effects on plant cells that we observed with the full length toxin.

Northern Blots. Northern slot-blot analyses of 10 transformants are shown in Figure 4A; hybridization intensities of sample RNAs are correlated with known quantities of synthetic *B.t.* toxin mRNAs (see "Materials and Methods"). Some of the insect-resistant plants contained less than 2 pg toxin-related RNA per 20 μg total cellular RNA, while the plant with highest expression (No. 857) showed approximately 47 pg toxin-RNA per 20 μg total RNA. This represents a 50-fold range of expression in hornworm-resistant transformants. Plants No. 858 and No. 884 showed evidence of toxin-related RNAs despite having only broken insertions; we have not analyzed this further since only marginal insect resistance was observed in either plant (below, and Table I).

Most transformants analyzed on slot-blots for both NPT-II and toxin RNAs contained approximately 10-fold more NPT-II hybridizing RNA, on a per weight basis (data not shown). However, northern gel analysis indicated a far greater disparity between the levels of the two mRNAs of the predicted sizes, 1230 bases for NPT-II, and 2230 bases for the toxin, excluding polyadenylation (Fig. 5, A and B). While the NPT-II mRNA generally appeared to be intact and of the predicted size in all plants analyzed, the toxin RNA routinely appeared as a minor species of the predicted size, with additional significant hybridization of distinct shorter fragments. Some of the shorter fragments co-migrated with less than full-length fragments of the standard *B.t.* RNA, which was generated by *in vitro* transcription using SP6 polymerase (see "Materials and Methods"). This suggests that the toxin RNA might have one or more regions that are particularly labile. Mapping of the additional toxin-related RNAs to determine their identity is now in progress, with both *in vivo* and *in vitro* synthesized transcripts serving as substrates.

Immunoblots. Immunoblot analysis of toxin-related polypeptides in plants No. 859 and No. 860 is shown in Figure 6. A specific immunoreactive polypeptide of approximately 72,000 D co-migrates with the truncated toxin standard (*tac B.t.*). Several additional protein bands of slower mobility were also present in the plant extracts. Control plant tissues H425, not transformed with the toxin chimera, do not contain either the 72,000 D or the higher mol wt polypeptides, although other more rapidly migrating cross-reacting polypeptides were detected in varying amounts in transformants and controls. The immunoreactive polypeptides of higher mobility observed in extracts of plants No. 859 and No. 860 are likely to be aggregates of the 72,000 D toxin, since we have occasionally observed similar bands in our *E. coli*-produced toxin extracts. Based on staining intensities and comparison with *E. coli*-produced toxin, we estimate that ap-

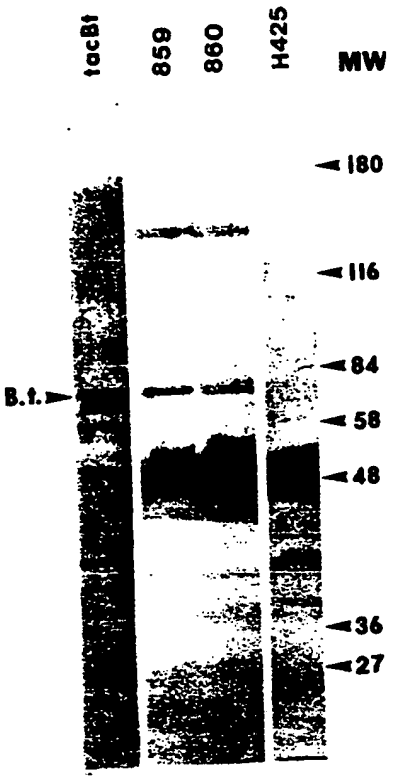

FIG. 6. Immunoblot analysis of AMVBTS plants. Total protein (80 μg) from plants No. 859, No. 860, and untransformed H425, and standard *B.t.* toxin generated in *E. coli* (*tac B.t.*) was electrophoresed on 7% polyacrylamide gels, then transferred to nitrocellulose. The blots were incubated with rabbit-anti-*B.t.* toxin serum, followed by alkaline-phosphatase-conjugated anti-rabbit immunoglobulin. Enzymic visualization of immunoreactive polypeptides was then carried out. The *E. coli*-produced truncated toxin band (*B.t.*) noted in lane 1 is estimated at approximately 5 ng, based on dye staining, immunoreactivity, and bioassays prior to dilution.

proximately 1 ng toxin protein per 80 μg total protein was present in extracts of these plants.

Analysis of Progeny. Table I presents information pertaining to larval toxicity, gene copy number, and amount of toxin mRNA in 10 regenerated transformants. A correlation between the level of toxicity in bioassays and the molecular characteristics of the toxin gene in the plants is apparent. Plants No. 858 and No. 884, both without intact toxin genes, do not show significant toxicity. The remaining eight plants were lethal to all the test larvae within the test period, and the highest toxicities were correlated with the highest levels of toxin-related transcripts.

Transmission of resistance to progeny was tested by allowing plants to flower, and seed was generated by self-pollination. Figure 3 (B and C) shows the results of Southern blot analyses of 10 seedlings derived from self-pollination of plant No. 857, which was found by additional restriction mapping to contain three independent insertions of the toxin chimera. The restriction digest generating internal toxin gene fragments (Fig. 3B) showed that nine of the progeny had one or more copies of the toxin gene. Additional restriction mapping, including border digests (Fig. 3C), revealed that various combinations of the three inserts were found in the progeny from plant No. 857. Figure 4B provides evidence that variable levels of toxin-related RNA appeared in progeny. The presence of toxin-related RNAs corresponded to the presence of one or more of the three toxin inserts in the progeny. However, the three inserts were not expressed at identical levels, since only marginal toxicity and little toxin-related RNA was seen when the insert characterized by the 1.5 kb border fragment (Fig. 4C) was the only insert present. Table II summarizes the data on insect bioassays and nucleic acid analyses for the progeny of plant No. 857. Similar analyses of progeny from additional AMVBTS plants have indicated that the AMVBTS gene routinely continues to express in progeny. Outcrosses are now in progress between various transformants and progeny to determine to what extent gene dosage regulates the level of toxin expression.

Toxicity Against Other Insects. The tobacco hornworm larvae are convenient for assays of toxicity because they are more sensitive to *B.t.* toxin than many other Lepidopteran insects, and are easy to use in a laboratory setting. However, to assess the practicality of the resistance obtained with the AMVBTS gene, we tested the resistance of toxin-producing plants to *H. zea*, *H. virescens*, and *S. exigua*. In four successive tests, using either the parent plant No. 857 or its progeny with all three insertions represented (No. 1265, for example), reductions in feeding and increased mortality of each larval type were observed relative to larvae fed control H425 tissues (data not shown).

DISCUSSION

We have generated transgenic tobacco plants that express a chimeric *B.t.* toxin gene at sufficient levels to provide resistance to Lepidopteran insects. Similar results have recently been reported (10, 27) using a crystal protein gene homologous to the one used in the present work, but differing in DNA sequence. Our results and those presented elsewhere (10, 27) indicate that the *B.t.* toxin presents unusual problems that must be overcome to obtain useful levels of toxin protein in plants. Our initial experiments led to the conclusion that expression of intact protoxin was in some way toxic to plant tissues, since transformants that contained toxin protein soon died, and no regenerated healthy transformants contained the toxin. While the truncated toxin did not show the same apparent toxicity toward plant cells, the level of toxin mRNA obtained in our insect-resistant transformants remained quite low relative to the adjacent NPT-II gene, and relative to other chimeric genes. The low level of toxin-related RNA correlates with low levels of toxin protein.

Table II. *Toxicity in Progeny of AMVBTS Plant No. 857*

Seeds resulting from self-pollination of AMVBTS plant No. 857 were germinated, and randomly selected seedlings were tested for AMVBTS insertions present (Fig. 3, B and C), toxin-related RNA content (pg/20 μg; see Fig. 4B), and for both feeding reductions relative to control tissues H425 (− to ++++) and larval mortality (no. killed/no. tested). The three possible genes in No. 857 progeny, based on border-mapping (Fig. 3C) are 'a' (3.8 kb border), 'b' (2.0 kb), and 'c' (1.5 kb).

Plant No.	Genes	RNA	Toxicity
H425	0	ND[a]	− (0/26)
1262	c	ND	− (3/6)
1263	c	ND	− (0/6)
1264	—	ND	− (0/6)
1265	a, b, c	6	++++ (6/6)
1266	a, b, c	6	++++ (6/6)
1267	a, b, c	5	++++ (6/6)
1268	a, b, c	12	++++ (6/6)
1269	c	ND	− (2/6)
1270	c	ND	− (4/6)
1271	c	ND	− (0/6)
1272	a, b, c	8	++++ (6/6)
1273	c	ND	− (2/6)
1274	a, b, c	24	++++ (6/6)
1275	c	ND	− (4/6)
1276	a, b, c	15	++++ (6/6)

[a] Not detectable.

However, due to the high potency of the *B.t.* toxin toward susceptible insects, some transformants were resistant to *M. sexta* even when toxin protein was below detectable levels.

We have introduced several unique features into the toxin gene to obtain useful levels of expression. These include a 5′ noncoding region corresponding to that of the AMV coat protein mRNA (13) and the addition of new codons at the point where the toxin coding region was artificially terminated. The utility of these features is undergoing further analysis. No accurate comparisons to alternative constructions are yet possible, since expression of chimeras prior to these modifications was too low to be detected. Our use of a plant virus noncoding region on the AMVBTS gene chimera was based on reports (11, 13) that plant viral mRNAs are exceptionally efficient in translational initiation.

Our results indicate that expression of the AMVBTS gene, based on steady state transcript levels, varies by more than 50-fold between independently derived insect-resistant transformants. Expression of the selectable marker, NPT-II, in the same transformants is consistently higher by approximately 10-fold. While the nopaline synthase promoter on the selectable marker would normally be considerably weaker than the CaMV 35S promoter (19), our positioning of the 35S enhancer between the promoters would likely provide a bidirectional enhancement equalize the promoter strengths. We cannot rule out the possibility that two promoters in such close proximity interact or compete for expression, but analogous constructions with reporter genes have previously shown active expression from both promoters. To compound the problem of low levels of *B.t.* toxin-related RNA the use of slot blots to quantitate toxin-specific mRNA was found to be deceptive. Northern gel hybridization analysis demonstrated that much of the toxin-related RNA was present in a low mol wt form, in RNA preparations where the NPT-II mRNA appeared quite intact. This suggests that the toxin transcript is unstable, possibly due to inefficient posttranscriptional processing or rapid turnover. We are exploring each of these possibilities by direct analysis of the toxin transcripts in plant cells and by synthetic modifications of the toxin coding sequence.

The levels of toxin present in the most actively expressing plants shown here were sufficient to demonstrate for the first time that extracts of resistant plants contain a peptide of the same size as the truncated toxin. These levels of toxin production are also sufficient to deter feeding patterns of various Lepidopteran pests, including the corn earworm, cotton bollworm, and beet armyworm. A detailed analysis of the present toxin gene expression, coupled with systematic alterations in primary and secondary characteristics of the transcript, will provide insight into problems specific to the expression of toxin protein in plants, as well as more generally to the expression of additional chimeras. Information resulting from such analysis is currently being applied to obtain cotton (26) resistant to Lepidopteran insects.

Acknowledgments—The authors are indebted to Michael Miller, Mark Maffitt, Sandra Cannon, Joseph Burkholder, Barry Cohen, and Edward Pahuski for expert technical assistance, and to Paul Christou, Will Swain, Paul Umbeck, and Winston Brill for many useful collaborations and discussions. We are also particularly grateful for support from the following at Cetus Corporation: Corey Levenson and his co-workers for oligonucleotide synthesis; Michael Innis and his staff members Ken Myambo and Mary Ann Brow for DNA sequencing; Sheng-Yung Chang for plasmid construction; and David Gelfand and Shing Chang for many productive interactions.

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EXHIBIT B

Fischhoff *et al.* (1987)

"Insect Tolerant Transgenic Tomato Plants." Bio/Technology **5**: 807-813

INSECT TOLERANT TRANSGENIC TOMATO PLANTS

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The structure of an insect control protein gene from *Bacillus thuringiensis* var. *kurstaki* HD-1 was determined, and truncated forms of the gene that express a functional insecticidal protein were generated. Two of these truncated genes were incorporated into a plant expression vector for *Agrobacterium*-mediated transformation. Transgenic tomato plants containing the chimeric genes express the insect control protein gene. Such expression confers tolerance to lepidopteran larvae on the transgenic tomato plants and their progeny. These engineered tomato plants represent a significant step to increased selectivity, specificity and efficiency in insect control.

Bacillus thuringiensis (B.t.) is an entomocidal, spore-forming bacterium. Most strains of *B.t.* are specifically lethal to lepidopteran insects (e.g., larvae of moths and butterflies)[1]. The insecticidal activity of *B.t.* resides in a parasporal protein crystal that is formed upon sporulation[2]. Crystal/spore preparations of *B.t.* have been used as commercial insecticides in products such as Dipel® (Abbott Laboratories) for many years. The commercial *B.t.* insecticides are effective against more than 50 lepidopteran pest species[3]. In the lepidopteran-type *B.t.* strains, the crystal is composed of insect control protein (*B.t.* protein) subunits of approximately 130 kD[4]. Several genes encoding lepidopteran-type *B.t.* proteins have been isolated and their DNA sequences determined[5–11].

In the past few years, it has become possible to introduce and express foreign genes in plant cells, especially through the use of *Agrobacterium*-mediated transformation[12]. For many species, transgenic plants containing and expressing such foreign genes have been regenerated. This technology has recently been applied to the genetic engineering into transgenic plants of agronomically important traits such as tolerance to the herbicide Roundup®[13,14] and tolerance to plant viruses[15].

Chemical control of insect pests is estimated to cost more than $3 billion per year worldwide. Over $400 million is spent each year for control of lepidopteran pests in the U.S. alone. The genetic engineering of insect tolerance into crop plants is therefore a goal of significant interest to agricultural biotechnology. A logical approach to this problem is through the expression of insect control protein genes from *B.t.* (*B.t.* genes) in transgenic plants. In this report we describe the construction of chimeric genes containing truncated forms of a lepidopteran-type *B.t.* gene that have been engineered for expression in plants. These genes have been introduced into tomato cells and transgenic plants have been recovered. We demonstrate that the chimeric *B.t.* genes are expressed in tomato plants and that expression of the insect control protein gene confers insect tolerance on the transgenic plants and their progeny.

RESULTS

Sequence of a *B.t.* insect control protein gene. We have previously described the isolation and initial subcloning of a lepidopteran-type insect control protein gene from *B.t.* var. *kurstaki* HD-1 (*B.t.k.* gene)[16]. This gene was contained on a BamHI-PstI fragment of approximately 4.5 kilobase pairs (kb) in plasmid pMAP4 (Fig. 2). We have determined the nucleotide sequence of 3734 base pairs (bp) of *B.t.k.* DNA contained in pMAP4 including the entire protein encoding segment. As shown in Figure 1 the sequence determined contains an open reading frame of 3468 bp encoding a protein of 1156 amino acids with a predicted molecular weight of 130,690. This is in good agreement with the size of the protein estimated by polyacrylamide gel electrophoresis. As previously described, the *B.t.k.* gene contained in pMAP4 expresses an insect control protein (*B.t.k.* protein) that is lethal to lepidopteran larvae when expressed either in *E. coli* or in *Pseudomonas fluorescens*[16,17].

Several groups have reported nucleotide sequences for lepidopteran-type *B.t.* genes[5–11]. We have compared both the nucleotide sequence and the derived amino acid sequence of the insect control protein gene shown in Figure 1 with these other reported sequences. Based on this analysis, the gene described here is nearly identical to a recently reported gene also isolated from *B.t.k.* HD-1 and designated *kurhd1*[11]. Our *B.t.k.* gene and *kurhd1* differ by just nine nucleotides in the coding region. These nucleotide differences account for four amino acid differences between the proteins including a one amino acid deletion in *kurhd1* relative to our gene. Two groups have reported nucleotide sequences for an insect control protein gene from *B.t.*. var. *berliner* 1715[9,10]; these two reported *berliner* sequences differ from one another by only two amino acid changes. Interestingly, our *B.t.k.* gene encodes a protein nearly identical to that encoded by the *berliner* genes; it differs from the gene of Wabiko et al.[9] by four amino acids and from the gene of Hofte et al.[10] by six amino acids.

Others have reported detailed comparisons between the published lepidopteran-type *B.t.* gene sequences[9–11]. We

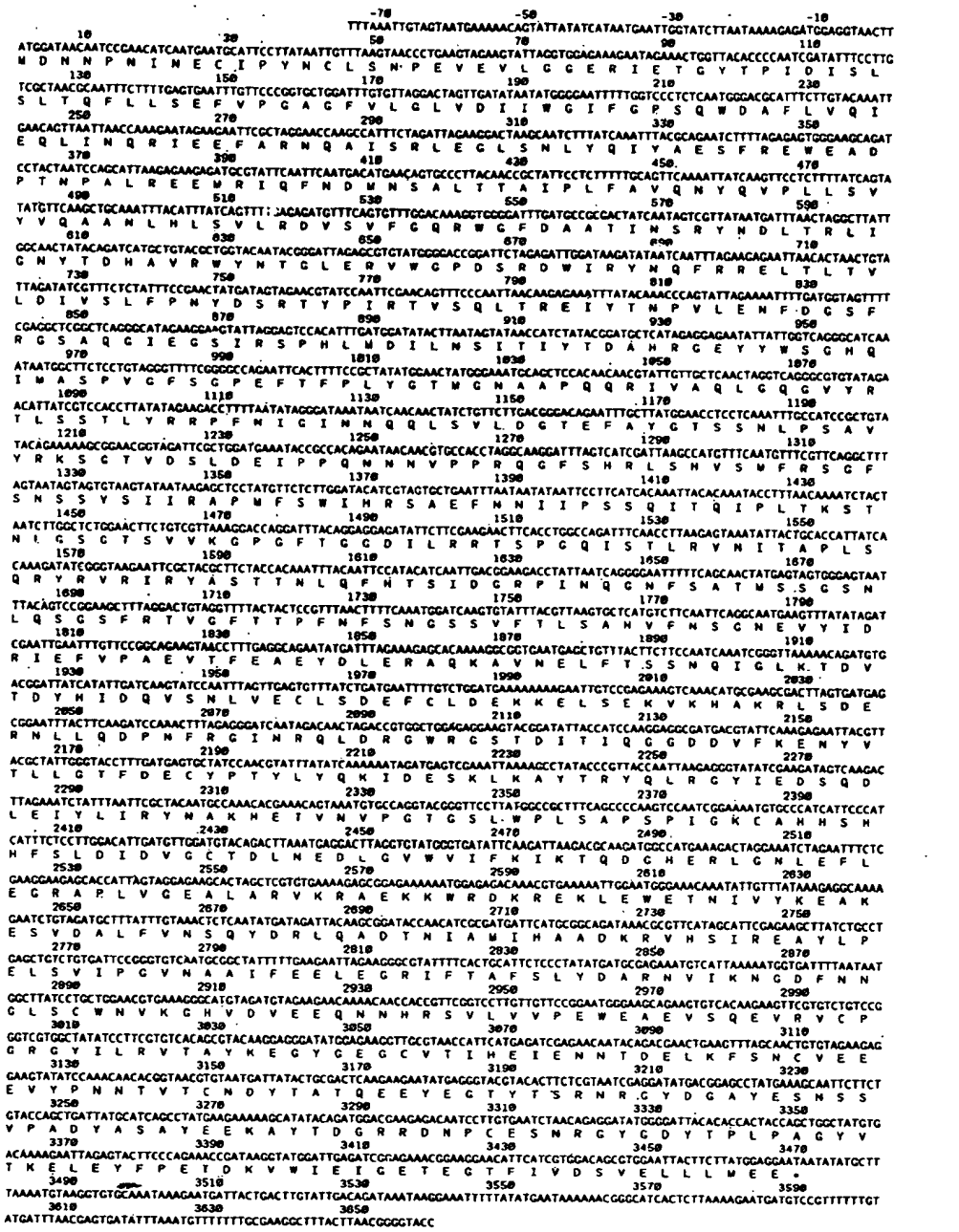

FIGURE 1 DNA sequence and derived amino acid sequence of the insect control protein gene from *B.t.* var. *kurstaki* HD-1. The sequence shown includes the entire *B.t.k.* protein coding sequence, 75 nucleotides of 5′ flanking sequence and 188 nucleotides of 3′ flanking sequence. Nucleotide coordinates are numbered from the first nucleotide of the translational initiation codon. The derived amino acid sequence is shown in the one letter amino acid code.

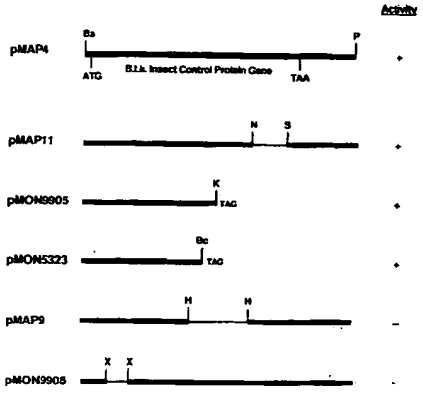

FIGURE 2 Deletion analysis of the *B.t.k.* insect control protein gene. The top line shows the structure of the 4.5 kb *B.t.k.* gene fragment in pMAP4. The initiation and termination codons for the protein are indicated. The deletion variants described in the text are shown below. The narrow lines indicate regions of the gene that have been deleted. Restriction enzyme cleavage sites are indicated as Ba, BamHI; Bc, BclI; H, HindIII; K, KpnI; N, NruI; S, ScaI; X, XbaI. The nucleotide coordinates of the indicated restriction sites are given in the text. pMON9905 and pMON5323 contain truncated *B.t.k.* genes fused to termination codons (indicated as TAG) provided by synthetic oligonucleotide linkers. The column on the right summarizes data on lethality to *M. sexta* larvae of the truncated *B.t.k.* proteins encoded by the variants.

have compared, in pairwise combinations, all of the published sequences, and our results confirm and extend these earlier reports (data not shown). In summary, the lepidopteran-type insect control proteins are highly homologous. However, they include a highly variable region that can extend from approximately amino acid 285 through amino acid 640. Based on differences in this variable region, the genes reported to date can be assigned to four classes. One class includes the *B.t.k.* gene described here, the *kurhd1* gene and the two *berliner* genes. A second class includes a gene from *B.t.* var. *sotto*[6] and the gene from *B.t.k.* HD-1 described by Schnepf et al.[5]. A gene from *B.t.k.* HD-73[7] forms the third class, and the fourth class contains another gene from *B.t.k.* HD-1 described by Thorne et al.[8].

Deletion analysis of the *B.t.k.* gene. It is known that proteolytic fragments of *B.t.* proteins, which are only about half the size of the intact protein, are active against insects[18,19]. In order to define shorter segments of the *B.t.k.* gene, which would still encode this functionally active domain, we have created a variety of deletion variants. These variants have been expressed in *E. coli*, and in all cases accumulation of protein of the expected size was observed by Western blot analysis utilizing antibodies prepared against purified crystal protein (data not shown). *E. coli* cultures expressing the deletion variants have been assayed for insect control activity by bioassay with neonate larvae of *Manduca sexta* (tobacco hornworm). The deletion variants analyzed and the results of the bioassay are shown in Figure 2.

The *B.t.k.* gene in pMAP11 contains a deletion extending from a NruI site at nucleotide 2704 to a ScaI site at nucleotide 3376 (nucleotide coordinates refer to the numbering in Fig. 1). This deletion causes an in-frame fusion and encodes an insect control protein lacking amino acids 902 to 1126. When expressed in *E. coli* this protein was lethal to *M. sexta*. pMON9905 contains a *B.t.k.* gene truncated at the KpnI site at nucleotide 2174. A synthetic oligonucleotide linker with two in-frame termination codons has been inserted in the KpnI site. The protein produced by this gene contains the first 725 amino acids of the intact insect control protein, and it was lethal to *M. sexta*. The *B.t.k.* gene in pMON5323 is truncated at the BclI site at nucleotide 1935 and also has a termination codon linker attached at this site. This truncated gene encodes a protein containing the first 646 amino acids of the intact insect control protein plus a four amino acid C-terminal extension encoded by the linker. This protein was also lethal to *M. sexta*. pMAP9 was created by deletion of the HindIII fragment extending from nucleotide 1692 to nucleotide 2747. The gene created by this manipulation encodes the first 565 amino acids of the intact insect control protein plus a three amino acid C-terminal extension created by the deletion. This truncated *B.t.k.* protein had no activity against *M. sexta*. One additional variant (pMON9906) was created by deletion of the XbaI fragment extending from nucleotide 292 to nucleotide 667. This deletion causes an in-frame fusion and encodes a protein lacking amino acids 98 through 222. This protein was not active in the bioassay.

Based on the analysis described above, the N-terminal half of the *B.t.k.* protein is essential for lethal activity, but the C-terminal half is dispensable. A truncated protein containing just the first 646 amino acids was a functional insect control protein. However, a protein containing the first 565 amino acids was not active. In addition, a deletion of 125 amino acids in the N-terminal portion of the protein (pMON9906) was sufficient to eliminate lethal activity. These results are consistent with the results obtained from similar deletion analysis performed on other lepidopteran-type *B.t.* genes[6,7,9,10,20].

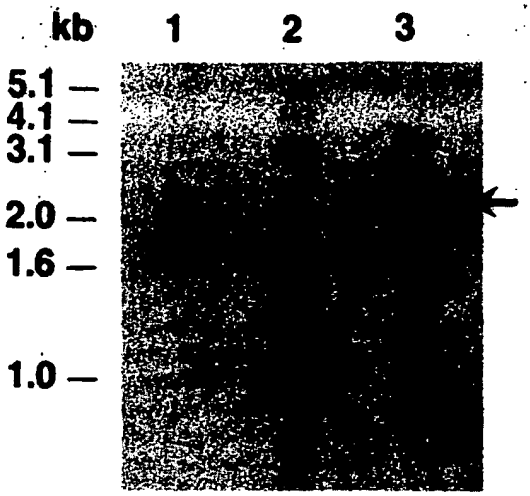

FIGURE 4 Northern hybridization analysis of *B.t.k.* mRNA in a pMON9711 transgenic plant. RNA was isolated from callus derived from leaves of pooled kanamycin resistant R1 progeny of plant #337, a pMON9711 transformant. The RNA was fractionated on a formaldehyde-containing agarose gel and blotted to a GeneScreen membrane. The membrane was hybridized with ^{32}P-labeled 4.5 kb BamHI-PstI fragment from pMAP17 containing the entire *B.t.k.* gene. Lane 1, *B.t.k.* gene DNA marker, the 2.2 kb BglII fragment from pMON9711 (2 ng). Lane 2, ^{32}P-labeled 1 kb ladder (Bethesda Research Laboratories). Sizes of the ladder fragments are indicated on the left. Lane 3, Poly A$^+$ RNA (25 μg) isolated from callus as described above. The arrow to the right indicates the position of the hybridizing *B.t.k.* RNA in lane 3.

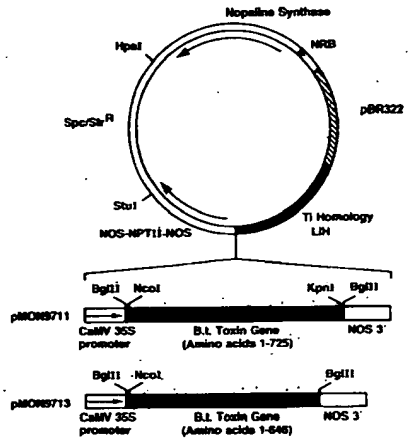

FIGURE 3 Plant transformation vectors containing chimeric truncated *B.t.k.* insect control protein genes. The structure of the chimeric *B.t.k.* gene contained in each vector is indicated. The CaMV 35S promoter is derived from cauliflower mosaic virus. Polyadenylation signals are derived from the nopaline synthase gene (NOS 3'). Important functional regions of the plant transformation vector are shown in the circular diagram above. NOS-NPTII-NOS, a chimeric gene conferring kanamycin resistance on plant cells; Spc/Str^R, a bacterial spectinomycin/streptomycin resistance gene; pBR322, the origin of replication from pBR322; the nopaline syntase gene from the Ti plasmid; NRB, the right border of the T-DNA from a nopaline Ti plasmid; LIH, a segment of the octopine Ti plasmid that provides a region of homology for cointegrate formation with disarmed Ti plasmids.

Plant transformation vectors containing truncated *B.t.k.* genes. Chimeric *B.t.k.* genes containing the CaMV 35S promoter, and either the full length *B.t.k.* protein coding sequence or the coding sequence for active truncated variants as defined by the deletion analysis, were constructed for expression in plants. The frequency at

which we were able to recover transgenic plants that exhibited detectable expression (by bioassay) of the chimeric *B.t.k.* gene was significantly greater when truncated genes were employed than when the full length gene was used. Initially, two transgenic tomato plants transformed with pMON9711 (which contains a truncated *B.t.k.* gene, Fig. 3) were recovered. When assayed for insect control activity, both of these plants displayed a high level of activity against *M. sexta* larvae (100% mortality in repeated bioassays). At the same time we recovered and assayed three transgenic plants containing a full length chimeric *B.t.k.* gene. Only one of these three plants had detectable activity, and the level of activity (50% to 80% mortality in repeated bioassays) was lower than for the plants containing the truncated gene. We thus focused our attention on the transgenic plants containing the truncated chimeric genes, and these results are described below.

Chimeric truncated *B.t.k.* genes were constructed for expression in plants as follows. The active truncated deletion variants contained in pMON9905 and pMON5323 were further modified by addition at their 5′ ends of a linker containing a BglII site (see Experimental Protocol). These truncated insect control protein genes were moved as BglII fragments into the BglII site of pMON316, a plant expression cassette vector[21]. pMON316 is a plant transformation vector based on pMON200; it contains an expression cassette consisting of the 35S promoter of cauliflower mosaic virus (CaMV) and the 3′ end of the nopaline synthase (NOS) gene from the Ti plasmid of *Agrobacterium* with a BglII site between these two elements. The resulting plant transformation vectors, pMON9711 and pMON9713, containing truncated chimeric *B.t.k.* genes are shown in Figure 3. These vectors were integrated into the disarmed Ti plasmid of *Agrobacterium tumefaciens* for transformation of plants[22].

Expression of *B.t.k.* genes in tomato plants. *Agrobacterium* strains containing pMON9711 and pMON9713 were used to transform tomato explants[23], and transgenic tomato plants containing the *B.t.k.* genes were recovered at a frequency of approximately three kanamycin resistant plants per one hundred explants inoculated with *Agrobacterium*. Expression of the *B.t.k.* gene contained in pMON9711 was assayed in one such plant by Northern hybridization analysis. As shown in Figure 4 (lane 3) this plant produced a polyadenylated mRNA that hybridized to a *B.t.k.* gene probe. The size of the mRNA estimated from this gel was approximately 2500 nucleotides. This is the expected size of a full length *B.t.k.* mRNA from pMON9711 given the size of the *B.t.k.* protein encoding BglII fragment and the known sites for transcriptional initiation from the CaMV35S promoter[24] and for polyadenylation by the NOS 3′ end[25]. For the experiment shown in Figure 4, RNA was isolated from callus derived from leaves of pooled kanamycin resistant R1 progeny of a transgenic plant (#337). We have detected a similar full length *B.t.k.* mRNA in leaves of this plant and an additional pMON9711 transformant; however, for R1 progeny of plant #337 the *B.t.k.* mRNA appeared to be more abundant in callus than in leaves. The hybridization analysis indicated that the expression level of the *B.t.k.* gene was quite low. The level of *B.t.k.* mRNA was substantially lower than the level of NPTII mRNA in these plants even though the NPTII gene is expressed from the nopaline synthase (NOS) promoter (Fig. 3) which is reported to be approximately 30-fold less active than the CaMV 35S promoter[21] driving the *B.t.k.* gene. In spite of the low abundance of *B.t.k.* mRNA, plants expressing the gene at this level are lethal to insects as described below.

Insect control activity of transgenic tomato plants against lepidopteran larvae. Tomato plants containing pMON9711 and pMON9713 have been tested for lethality to lepidopteran larvae utilizing either isolated leaves or whole plants in feeding assays. Figure 5 shows an example of the isolated leaf bioassay against larvae of *M. sexta*. As can be seen in the figure, after four days of feeding ten neonate larvae were able to consume an entire leaf from a nontransformed plant. There was no mortality, and the larvae increased in size approximately five-fold. In contrast, the larvae feeding on the pMON9711 transgenic plant were all killed within 48 hours, and there was very little evidence of feeding damage to the leaf. This lack of damage by larvae feeding on the transgenic plant is consistent with the known effects of *B.t.* insect control protein. One early sign of *B.t.* intoxication is an inhibition of feeding by larvae[26]. Similar lethality is seen at the whole plant level as illustrated in Figure 6. After seven days of feeding, ten *M. sexta* larvae were able to defoliate completely a six week old nontransformed tomato plant, and the larvae had begun to attack the stems as well as the leaves. Again there was no mortality. The larvae feeding on the pMON9711 transgenic plant were all killed within 72 hours and the plant showed very little evidence of feeding damage.

These results are not limited to the plants shown in the figures. We have recovered ten pMON9711 transgenic plants and five pMON9713 transgenic plants that show similar lethality to *M. sexta*. In the case of pMON9711, 17 kanamycin resistant transgenic plants have been assayed for insect control activity with the *M. sexta* bioassay. Ten of these plants were highly lethal to the larvae (90% to 100% mortality); two plants showed lower, but detectable, activity (20% to 50% mortality), and five had no detectable activity. For pMON9713, four of the five transgenic plants recovered had high activity. We have also tested the insect control activity of these transgenic plants with larvae of two other lepidopterans, *Heliothis zea* (corn earworm) and *Heliothis virescens* (tobacco budworm). Our studies with *E. coli* cultures expressing the cloned *B.t.k.* gene indicate that *M. sexta*, *H. virescens*, and *H. zea*, in that order, are increasingly difficult to kill (data not shown). The results of several typical plant assays are summarized in Table 1. Some of the transgenic plants were able to kill 100% of the *H. virescens* larvae tested, while other plants were not quite as effective. For most of the plants tested, the *H. virescens* larvae that were not killed were severely stunted compared to larvae feeding on control tissue. Typically, larvae on control leaves doubled in size during the four day assay period while larvae on the transgenic leaves showed no weight gain. To date we have tested only one plant against *H. zea*. The results indicate that, as expected, the mortality seen with this insect was somewhat less than that seen with *H. virescens;* however, severe stunting was again seen in all of the surviving larvae. No mortality has been seen in larvae feeding on control transgenic tomato plants transformed with vectors which do not contain a chimeric *B.t.k.* gene (see Table 1).

As is also indicated in Table 1, we have isolated first generation and second generation progeny of these transgenic plants including some putative homozygous progeny. The *B.t.k.* gene segregated with nopaline production and kanamycin resistance as expected. In all of the plants tested to date the *B.t.k.* gene segregated as a single dominant Mendelian marker indicating the presence of a single functional T-DNA locus. As shown in the table, the progeny plants are at least as lethal as the primary transformants, and sometimes display even greater lethality. The results presented here indicate that transgenic tomato plants containing either of the truncated chimeric *B.t.k.* genes are able to express the insect control protein at levels sufficient to kill lepidopteran larvae.

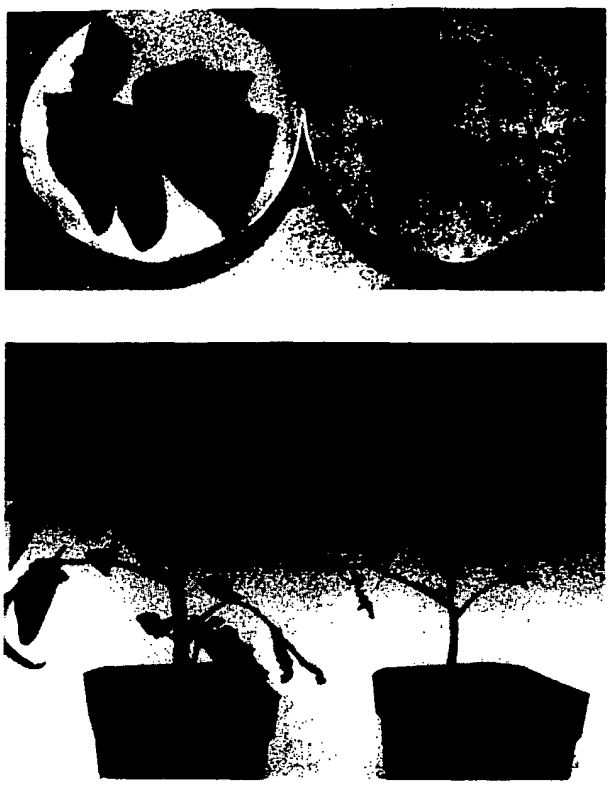

FIGURE 5 Insect bioassay of isolated leaves of a pMON9711 transgenic tomato plant. Isolated leaves were assayed for insect control activity against *Manduca sexta* larvae. Ten neonate larvae were applied to leaves and allowed to feed for four days. Left, leaves from a pMON9711 transgenic tomato plant (#337); dead larvae are visible on the leaf. Right, leaves from a nontransformed control plant; viable larvae are visible.

FIGURE 6 Whole plant insect bioassay of a transgenic tomato plant. Intact tomato plants were assayed for insect control activity against *Manduca sexta* larvae. Ten neonate larvae were allowed to feed on each plant for seven days. Left, a pMON9711 transgenic tomato plant. Right, a nontransformed control plant; viable larvae are visible.

TABLE 1 Toxicity of transgenic tomato plants to lepidopteran larvae.

Plant	Vector (pMON)	Corrected Mortality (%)		
		M. sexta	*H. virescens*	*H. zea*
337 (R0, R2)	9711	100	100	n.d.
344 (R0)	9711	100	2	n.d.
344 (R2)	9711	100	50–70*	10–50*
482 (R1)	9713	100	35–70*	n.d.
306 (R0, R2)	319	0	0	0
379 (R0)	200	0	n.d.	n.d.
Control	none	0	0	0

* Surviving larvae show severe stunting

Results of insect bioassays of pMON9711 and pMON9713 transgenic tomato plants with larvae of *Manduca sexta, Heliothis virescens* and *Heliothis zea*. Bioassays were performed as described in the Experimental Protocol. Results are also shown for control transgenic tomato plants transformed with pMON200[22] or pMON319[15] that do not contain chimeric *B.t.k.* genes and for a nontransformed plant. R0, primary transformed plants regenerated from tissue culture. R1, kanamycin resistant, first generation progeny from selfed R0 plants; a mixture of homozygous and heterozygous transgenic plants. R2, homozygous second generation kanamycin resistant progeny derived from selfed R1 plants.
n.d., not done.

In this report we show that expression of a lepidopteran specific insect control protein gene from *Bacillus thuringiensis* confers insect tolerance on transgenic tomato plants. Recently, others have reported that expression of similar *B.t.* insect control protein genes can confer insect tolerance on transgenic tobacco plants (M. Vaeck, personal communication; M. Adang, personal communication). In the experiments reported here we have utilized truncated forms of the gene encoding functional proteins. These genes were expressed from the CaMV 35S promoter, which is known to be a strong promoter in transgenic plants[21]. The chimeric truncated *B.t.k.* gene in pMON9711 was expressed as a full length polyadenylated mRNA. However, the level of *B.t.k.* mRNA was much lower than expected for a chimeric gene expressed from the CaMV 35S promoter. At this point it is not clear why the *B.t.k.* mRNA is not more abundant, but the low level of stable *B.t.k.* mRNA accumulation might indicate that the chimeric transcript is unusually unstable in plant cells. In spite of the low level of RNA expression, the insecticidal protein was expressed at a level sufficient to kill larvae of three important lepidopteran pests of tomato, namely, *M. sexta, H. virescens,* and *H. zea*[27]. The fact that insect control activity is seen even under conditions of low level gene expression is a reflection of the high potency of the *B.t.k.* protein. In the case of the *Heliothis* larvae, although some of the larvae survived four days of feeding on some of the plants tested, the growth of the surviving larvae was severely inhibited. The plants sustained little damage because larval feeding was significantly reduced. Therefore, expression of the protein at levels comparable to that found in the plants described here might provide agronomically significant control of insect pests. These effects, feeding inhibition and stunting of growth at sublethal doses, are known properties of the *B.t.k.* protein[26].

In addition to damaging the foliage of tomato plants, lepidopteran larvae, especially of the *Heliothis* species, can do considerable damage to tomato fruit, making the fruit unmarketable[27]. Newly hatched larvae typically feed on the foliage before feeding on the fruit, so *B.t.k.* expression in leaves might be sufficient to reduce or eliminate fruit damage. For complete protection it might be necessary to have the insect control protein expressed in the fruit as well. We have obtained preliminary evidence that some *B.t.k.* activity is detectable in tomato fruit from our transgenic plants. *Heliothis virescens* larvae fed on transgenic fruit gained weight at only one half the rate of larvae fed on fruit of a nontransformed plant, consistent with a low level of *B.t.k.* protein in the fruit.

We have not directly measured the amount of *B.t.k.* protein produced by these transgenic plants. However, it is possible to estimate the amount of protein produced by measuring the amount of leaf material consumed by the *M. sexta* larvae before they are killed. Based on the lethality of *E. coli* cultures expressing known amounts of the truncated protein encoded by the deletion variant in pMON9711 (data not shown), we estimate that the transgenic plants are producing on the order of 50 ng of *B.t.k.* protein per gram of leaf tissue (fresh weight). It should be possible to increase the level of protein production substantially through further manipulation of the chimeric genes, and we are currently investigating this possibility. We have seen differences in the level of expression of the *B.t.k.* gene from plant to plant as measured by the bioassay. These differences are consistent with the plant to plant variation seen with other chimeric genes in transgenic plants[21,28].

This report demonstrates the feasibility of genetically engineering insect tolerance into transgenic crop plants

through the expression of a *B.t.k.* insect control protein gene. Plants producing their own insecticidal proteins increase the selectivity of control since only pests that attack the plant will be affected. These plants also afford continuous control in contrast to the sporadic control provided by present application technology. Genetically engineered insect tolerant crop plants should prove to be a valuable addition to the strategies currently available for insect pest control.

EXPERIMENTAL PROTOCOL

DNA manipulations. Recombinant DNA techniques were as described by Maniatis et al.[29] DNA sequence analysis of the *B.t.k.* gene was performed by the dideoxy method of Sanger et al.[30] utilizing 80 cm buffer gradient gels[31] following subcloning of individual restriction fragments into M13 vectors mp8 and mp9[32].

Construction of deletion variants of the *B.t.k.* gene. pMAP9, pMAP11 and pMON9906 are simple deletions of restriction fragments from the *B.t.k.* gene in pMAP4. For construction of pMAP9, pMAP11 and pMON9906, pMAP4 was digested with NruI plus ScaI, HindIII and XbaI, respectively. Following digestion, the DNA was recircularized by treatment with T4 DNA ligase and transformed into *E. coli*. Appropriate clones containing the desired deletion variants were identified by restriction enzyme analysis. pMON9905 was constructed by digestion of pMAP4 with KpnI. Following digestion, the DNA was ligated with a synthetic oligonucleotide pair of sequence:

5'-CTAGTAAA-3'
3'-CATGGATCATTTCTAG-5'.

This synthetic linker contains a KpnI overhang and two termination codons in frame with the *B.t.k.* protein coding sequence at the KpnI site at nucleotide 2174. In addition, when ligated to itself, the linker creates a BglII site. Following transformation of *E. coli*, a clone containing the desired deletion and a BglII site was identified by restriction enzyme analysis and designated pMON9905. pMON5323 was constructed in similar fashion except that the *B.t.k.* gene was first digested with BclI. After digestion the single-stranded ends were made flush by treatment with DNA polymerase I Klenow fragment. The DNA was then ligated with a synthetic oligonucleotide pair of sequence:

5'-TAGTAGGTAGCTAGCCA-3'
3'-ATCATCCATCGATCGGTCTAG-5'.

This synthetic linker contains termination codons in all three reading frames. In addition, when ligated to itself, the linker creates a BglII site. When ligated to pMAP4 DNA treated as described above, this linker creates a gene which encodes 646 amino acids of the *B.t.k.* protein plus three amino acids encoded by the linker. Following transformation of *E. coli*, a clone containing the truncated gene and a BglII site was identified by restriction enzyme analysis and designated pMON5323.

Construction of plant transformation vectors containing truncated *B.t.k.* genes. An NcoI site was engineered at the ATG initiation codon of pMAP4 by site-specific[33] mutagenesis to create plasmid pMAP17. The 5' end of the gene was further modified by addition of a synthetic oligonucleotide of sequence: 5'-GGATCCAGATCTGTTGTAAGGAGTCTAGA-3' just 5' to the NcoI site to create plasmid pMON294. This linker contains a BglII site (underlined). To construct a plasmid vector for plant transformation containing a *B.t.k.* gene truncated at the KpnI site at nucleotide 2174, pMON294 was digested with KpnI, ligated with the synthetic linker utilized in the construction of pMON9905 described above and then digested with BglII. A 2.2 kb BglII fragment containing the truncated gene was isolated and ligated with BglII digested pMON316[21]. A clone in which the 5' end of the *B.t.k.* gene was adjacent to the CaMV 35S promoter was isolated and designated pMON9711. To construct a plasmid containing a *B.t.k.* gene truncated at the BclI site at nucleotide 1935, pMON294 was digested with BclI, treated with DNA polymerase I Klenow fragment, ligated with the synthetic linker utilized in the construction of pMON5323 described above and then digested with BglII. A BglII fragment of approximately 2.0 kb containing the truncated gene was ligated with BglII digested pMON316. A clone in which the 5' end of the *B.t.k.* gene was adjacent to the CaMV 35S promoter was isolated and designated pMON9713.

***Agrobacterium*-mediated transformation and regeneration of transgenic plants.** Plant transformation vectors pMON9711 and pMON9713 were introduced into *Agrobacterium tumefaciens* strains containing disarmed Ti plasmids as described by Fraley et al.[22] The *Agrobacterium* strains containing these vectors were used to transform explants of tomato *(Lycopersicon esculentum)* line VF36, and transgenic tomato plants were recovered as described by McCormick et al.[23] Primary transgenic plants regenerated from tissue culture were designated R0. The R0 plants were selfed, and first generation progeny plants were designated R1. R1 plants were assayed for inheritance of the transforming DNA by scoring for nopaline synthesis and for kanamycin resistance. R1 plants, which had inherited the transforming DNA, were selfed and the second generation plants were designated R2. Putative homozygous R1 plants were identified as those that did not segregate any nopaline negative, kanamycin sensitive progeny in the R2 generation.

RNA filter hybridization. Total RNA was isolated from tomato tissue[34], and poly A^+ RNA was purified by oligo-dT cellulose chromatography. Poly A^+ RNA was fractionated on agarose gels containing formaldehyde[29] and blotted directly onto GeneScreen membranes in 10× SSC buffer. Blots were hybridized with a ^{32}P-labeled BamHI-PstI restriction fragment from pMAP17, which contains the entire *B.t.k.* gene. Hybridization was at 42 °C in a solution containing 50% formamide, 5× SSC, 5× Denhardt's reagent, 1% SDS and 100 μg/ml salmon sperm DNA. The final posthybridization wash was at 50 °C in 0.1× SSC, 0.2% SDS.

Insect bioassays. *E. coli* cultures expressing the *B.t.k.* gene were assayed for lethality to neonate *Manduca sexta* (tobacco hornworm) larvae by incorporation into an artificial diet as previously described[17]. Tomato plants containing the *B.t.k.* gene were tested for lethality to *M. sexta* using both an isolated leaf feeding assay and a whole plant feeding assay. In the isolated leaf assay, one or more tomato leaves from a single plant were excised and placed in a Petri dish on water saturated filter paper. Ten neonate larvae were applied to the leaves and allowed to feed for four days at which time they were scored for mortality and weight gain. For the whole plant assay, ten neonate larvae were applied to the leaves of tomato plants (typically four to eight weeks after planting) and allowed to feed for four to seven days. At the end of the assay the larvae were scored for mortality and weight gain, and the plants were rated for amount of damage. Transgenic tomato plants were also assayed for lethality to *Heliothis virescens* (tobacco budworm) larvae and to *Heliothis zea* (corn earworm) larvae in a modified isolated leaf feeding assay. Individual leaves were placed in Petri dishes, and only a single three to four day old larva (2nd or 3rd instar) was added to each dish. After four days the larvae were scored for mortality and weight gain. A total of ten to twenty *Heliothis* larvae were tested on isolated leaves from each plant. All insect bioassays were performed in duplicate or triplicate. Corrected mortality was calculated according to Abbott's formula[35].

Received 8 May 1987; accepted 26 May 1987.

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EXHIBIT C

Vaeck *et al.* (July 2, 1987)

"Transgenic plants protected from insect attack." Nature **327**(6125): 33-37

Transgenic plants protected from insect attack

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The Gram-positive bacterium Bacillus thuringiensis *produces proteins which are specifically toxic to a variety of insect species. Modified genes have been derived from* bt2, *a toxin gene cloned from one* Bacillus *strain. Transgenic tobacco plants expressing these genes synthesize insecticidal proteins which protect them from feeding damage by larvae of the tobacco hornworm.*

MODERN agriculture uses a wide variety of insecticides to control insect damage. Most of them are chemically synthesized. Notable exceptions are the insect toxins produced by *Bacillus thuringiensis*: spore preparations of this Gram-positive bacterium have been used for more than 20 years as a biological insecticide[1]. The insecticidal activity resides in crystalline inclusion bodies produced during sporulation of the bacteria, which are composed of proteins (termed delta endotoxins) specifically toxic against a variety of insects. Different strains of *B. thuringiensis* differ in their spectra of insecticidal activity. Most are active against Lepidoptera, but some strains specific to Diptera[2,3] and Coleoptera[4,5] have been identified. The crystals dissolve in the alkaline conditions of the insect midgut and release proteins of relative molecular mass 65,000–160,000 (M_r 65K–160K)[2,5,6] which are proteolytically processed by midgut proteases to yield smaller toxic fragments[7]. *B. thuringiensis* insect toxins are highly specific, in that they are not toxic to other organisms. Hence, they are safe insecticides and present an interesting alternative to chemical control agents. Their commercial use however is limited by high production costs and the instability of the crystal proteins when exposed in the field.

We have used *Agrobacterium*-mediated T-DNA transfer[8] to express chimaeric *B. thuringiensis* toxin genes in tobacco plants with the objective of protecting the plants from insect attack. We show here that a defence mechanism against phytophagous insects can be devised by genetic engineering.

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Modified *Bacillus* toxins

We have reported[9] the cloning of the *bt2* gene from *B. thuringiensis* strain berliner 1715 and the characterization of the recombinant polypeptide expressed in *Escherichia coli*. This protein, termed Bt2, is 1,155 amino acids long and is a potent toxin to several lepidopteran larvae, such as those of *Manducta sexta*, a pest of tobacco. Bt2 is a protoxin and generates a smaller polypeptide of M_r 60K which retains full toxic activity[9]. The smallest fragment of Bt2 that is still fully toxic has been mapped in the NH_2-terminal half of the protein, between amino-acid positions 29 and 607 (ref. 9) (and see Fig. 1*a*).

In plant transformation experiments, we used chimaeric genes containing the entire coding sequence of *bt2* as well as truncated genes. A diagram of the chimaeric genes is shown in Fig. 1*b*. Some of our T-DNA constructs include a chimaeric neomycin phosphotransferase gene (*neo*) as a marker selectable in plants[10]. Others carry translational fusions between fragments of *bt2* and the *neo* gene. Fusions to the 5′ end of the *neo* gene still confer kanamycin resistance in bacteria[11] and in plants[12]. Plasmid pLB884 (ref. 9) contains the truncated gene *bt884* and encodes an NH_2-terminal fragment of Bt2 up to amino-acid position 610. In *E. coli* it produces a polypeptide of the expected size which is fully toxic towards insect larvae[9].

Plasmids pLBKm860 and pLBKm23 (H.H. *et al.*, in preparation) contain fusion genes *bt*:*neo860* and *bt*:*neo23* encoding NH_2-terminal fragments of Bt2 up to amino-acid position 683 and 724, respectively. *E. coli* cells harbouring these plasmids are resistant to kanamycin and produce fusion proteins Bt:NPT860 and Bt:NPT23 which have the expected M_r of 106K.

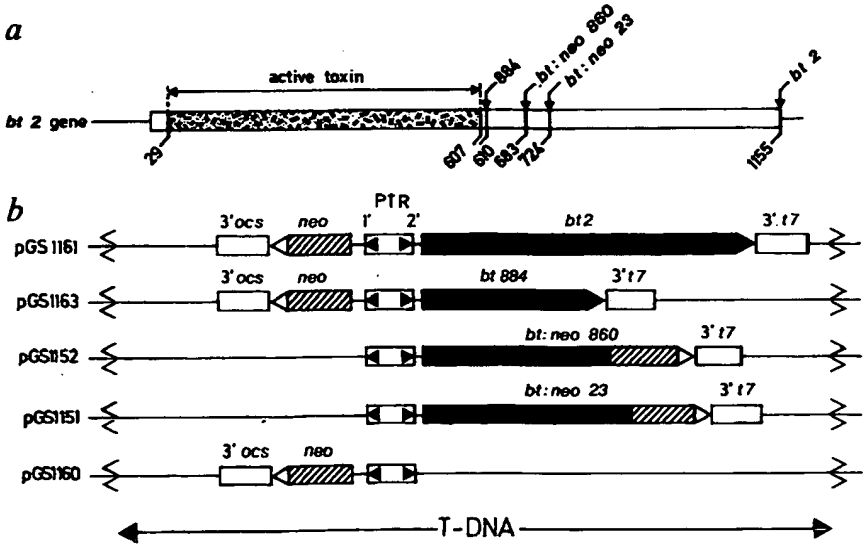

Fig. 1 *a*, Structure of the *bt2* gene. The smallest gene fragment encoding an active insect toxin is indicated, and the 3′ end positions (codon numbers) of the different truncated genes. *b*, Chimaeric genes derived from *bt2* present in plant expression vectors. The 5′ end of the *bt2* coding sequence is fused to the 2′ promoter fragment of the TR DNA. pGS 1161 contains the intact *bt2* gene. The *bt2* segment in pGS1163 ends at nucleotide position 1,830 of the *bt2* coding sequence. In pGS1151 and pGS1152, the *neo* gene has been fused to 5′ fragments of the *bt2* gene at positions 2,173 and 2,050 of the *bt2* coding sequence, respectively. PTR, a 482-base pair (bp) fragment containing the TR DNA 1′ and 2′ promoters, isolated from pOP443 (ref. 15); 3′t7, a 211-bp fragment containing the polyadenylation site of the TL DNA gene 7 (ref. 23); 3′ocs, a 706-bp *Pvu*II fragment containing the polyadenylation site of the octopine synthase gene[24]. Plasmids pGS1160, pGS1161 and pGS1163 were made using the intermediate plasmid pGSH160 which contains a chimaeric *neo* gene[14]. For pGS1151 and pGS1152, containing the *bt2*:*neo* fusion genes pGSH150, a derivative of pGSH160, lacking the *neo* gene and 3′ ocs, was used.

and 110K respectively and which react with anti-Bt2 (Fig. 2A) and anti-NPTII antibodies. At least 50% of the fusion proteins is present in a soluble form in the bacterial cells. The neomycin phosphotransferase activity of the fusion proteins, as determined in an *in situ* assay[13], is comparable to wild-type NPTII activity. Little or no enzymatic activity was exhibited by polypeptides of lower M_r (Fig. 2*b*). We conclude that the fusion proteins are relatively stable and responsible for the kanamycin-resistant phenotype. Insect assays revealed that, on a molar basis, the fusion proteins exhibit the same toxicity towards *M. sexta* larvae as intact Bt2 protein which has an LD_{50} value on first instar larvae of 4 ± 2 ng per larva.

Plant expression vectors

The intact and modified *B. thuringiensis* toxin genes were inserted between the T-DNA borders of plant expression vector pGSH160 (for *bt2* and *bt884*) or pGSH150 (ref. 14) (for *bt*: *neo860* and *bt*: *neo23*). These plasmids contain the promoter of the 2′ gene, a constitutive promoter which directs expression of mannopine synthase in the TR DNA of plasmid pTiA6 (ref. 15). The *Bacillus* genes are followed by a termination signal provided by the 3′ end of gene 7 of pTiA6. The resulting plasmids were mobilized into the *Agrobacterium* recipient C58C1 Rif^R pGV2260. The latter contains an octopine Ti plasmid from which the whole T-DNA region has been deleted and replaced by pBR322 (ref. 14). Recombination between pGV2260 and the expression vector through the homologous pBR322 sequences produced Ti plasmids pGS1161, pGS1163, pGS1151 and pGS1152 containing *bt2*, *bt884*, *bt*: *neo23* and *bt*: *neo860*, respectively (Fig. 1).

Two approaches were used to increase the probability of obtaining high levels of toxin expression in plants. First, the expression levels directed from the 1′ and 2′ promoters from the TR DNA were found to be coordinated[15]. Consequently we expected that expression of the *neo* gene controlled by the 1′ promoter in plants transformed with pGS1161 and pGS1163, would be correlated with transcription of the toxin gene. Second, we anticipated that plant cells transformed with pGS1151 or pGS1152, containing *bt*: *neo* fusions, would produce fusion proteins expressing NPTII activity. Selection for high levels of kanamycin resistance would allow us to select directly for transformed clones producing substantial amounts of *B. thuringiensis* protein.

Transformation of tobacco plants

Transgenic tobacco plants were obtained by leaf disk infection[14,16] of *Nicotiana tabacum* var. Petit Havana SR1 (ref. 17). Shoots resistant to 50, 100 or 200 $\mu g\ ml^{-1}$ kanamycin were selected in all transformation experiments, indicating that the fusion genes indeed confer NPTII activity on transformed plant cells. Individual transformed plants were grown up and subsequently assayed for kanamycin resistance by testing their ability to produce callus from leaf disks on increasing concentrations of kanamycin and the more toxic aminoglycoside antibiotic G418. Most of the transgenic plants expressing an intact NPTII protein produced highly resistant calli, growing on 1,000 $\mu g\ ml^{-1}$ kanamycin and on 100 $\mu g\ ml^{-1}$ G418. In contrast, plants that expressed a Bt: NPTII fusion protein readily fell into different classes of kanamycin resistance (Table 1). Because the specific enzymatic activity of the fusion proteins is comparable to that of intact NPTII, we presume that these fusion proteins are present in lower amounts in the plant cells than intact NPTII protein.

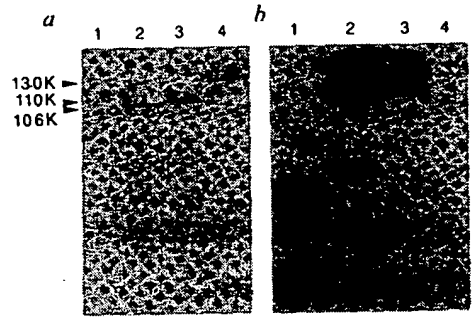

Fig. 2 Antigenic properties and enzymatic activity of Bt: NPTII fusion proteins. *a*, Western blot analysis[25] of crude extracts of *E. coli* clones producing intact NPTII enzyme (lane 1), Bt: NPT860 (lane 2) or Bt: NPT23 fusion protein (lane 3). Lane 4, purified Bt2. Blots were incubated with a diluted anti-Bt2 serum and subsequently with alkaline phosphatase-labelled anti-rabbit immunoglobulin. Substrates were 5-bromo-4-chloro-3-indolyl phosphate-toluidine salt and *p*-nitro-blue tetrazolium chloride

Insecticidal activity in transgenic plants

Leaves of transgenic plants containing the four types of *B. thuringiensis* gene constructs were fed to *M. sexta* larvae in order to evaluate whether the levels of toxin in the plants would be insecticidal. Mortality rates of *M. sexta* larvae were monitored after 6 days of feeding on leaves of transformed plants (Fig. 3). We found that high toxicity to insects, resulting in 75–100% mortality of the larvae, was observed in about one quarter of the plants that expressed the longer fusion protein Bt: NPT23 and in about two-thirds of those with the shorter Bt: NPT860. Thus, the Bt: NPTII fusions allowed us to select transformants that express levels of the toxin sufficiently high to be insecticidal. Second, insect toxicity caused by the fusion proteins is directly correlated with the level of kanamycin resistance of the transformed plant. In addition, the short fusion generates a larger fraction of transformants expressing high kanamycin resistance and insect toxicity than the longer fusion, suggesting that the shorter *bt*: *neo860* gene provides higher levels of biologically active protein than the longer *bt*: *neo23*. Clear insecticidal activity was also detected in most of the 15 plants expressing the truncated *bt884* gene, of which two-thirds induced more than 75% larval death. None of the plants transformed with the full length *bt2* gene produced insect killing activity above levels obtained in NPTII-expressing control plants. These experiments indicate that for the promoter gene constructs we used, only truncated *bt2* genes give rise to expression levels that are strongly insecticidal in transgenic tobacco.

Protection from insect damage

To test whether expression of modified *bt2* genes in plants results in effective protection against insect damage, selected transgenic plants were grown in the greenhouse and were infested with freshly hatched larvae of *M. sexta*. Plants were kept under conditions that were optimal for survival and growth of the insects. Plants N21-11 and N28-16 were highly protected, because the larvae stopped feeding within 18 hours and all were killed within three days. The damage caused by single larvae was limited to areas of only a few square millimetres (Fig. 4). Other plants, such as N21-53 or N28-6, suffered slightly more damage. On these plants however all the larvae were killed after six days. Control plants such as N21-110, transformed with pGS1160, or untransformed SR1, were severely damaged within 4–6 days and were entirely consumed after 12 days.

Expression of chimaeric genes in plants

Quantitative detection of *B. thuringiensis* toxin in the leaves of transformed plants was performed using a sensitive ELISA, with a mixture of monoclonal antibodies specific for the NH_2-terminal region of the protein. A correlation was found between the quantity of *Bacillus* protein and insecticidal activity in the transgenic plants. Plants transformed with the truncated *bt2* gene or the fusion constructs contain approximately ten times

Table 1 Kanamycin and G418 resistance in transgenic tobacco plants

Agrobacterium strain	Expression products of chimaeric genes	No. of plants resistant: Kanamycin ($\mu g\ ml^{-1}$) <50	50	100	200	500	1000	G418 ($\mu g\ ml^{-1}$) <10	10	100	Total
pGS1151	Bt:NPT23	3	1	7	17	14	10	51	1	0	52
pGS1152	Bt:NPT860	0	0	0	0	5	31	35	1	0	36
pGS1161	Bt2, NPTII	0	0	0	0	0	14	0	0	14	14
pGS1163	Bt884, NPTII	0	0	0	0	1	14	1	0	14	15
pGS1160	NPTII	0	0	0	0	2	12	2	0	12	14

Plants were scored according to the highest concentration of antibiotic on which callus could be induced from leaf disks from *in vitro* grown plants[21].

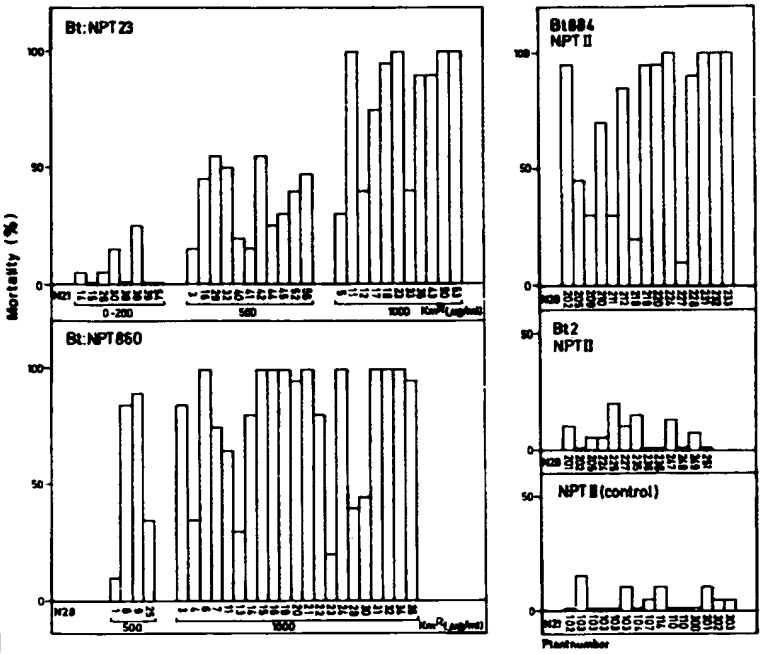

Fig. 3 Insect toxicity of transgenic tobacco plants expressing *B. thuringiensis* protein. Mortality of *Manduca sexta* larvae after 6 days of feeding on tobacco leaves is shown. The serial number of each plant is given below each column, and in the left-hand panels plants are grouped according to the concentration (in $\mu g\ ml^{-1}$) of kanamycin to which they are resistant. Each panel is labelled with the protein expressed (see Fig. 1). Plants were tested 4–6 weeks after transfer to the greenhouse, when they were about 40-cm high and had between six and eight full grown leaves. Two leaf disks of 4 cm in diameter were placed on wet filter paper in Petri dishes and infested with two batches of 10 first instar larvae of *M. sexta*. Leaf disks were replaced daily. Tests were conducted in a growth chamber at 25 °C, 75% relative humidity and under a 16 h light/8 h dark cycle.

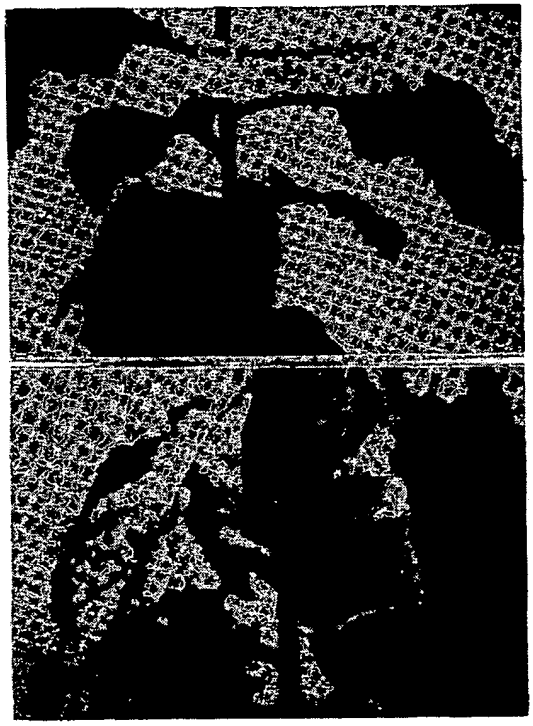

Fig. 4 Protection from insect feeding damage in transgenic plants expressing *B. thuringiensis* protein. Plants 40–50-cm high were infested in the greenhouse with fifteen *M. sexta* larvae per plant. *a*, On N21-11, expressing Bt:NPT23, all larvae died within three days. Leaf damage is very limited. *b*, Damage on a control plant expressing NPTII. Pictures were taken after 11 days.

inefficient protein synthesis in these transformed plant cells.

Transgenic plants that express the shorter Bt:NPT860 protein are on average more effective in killing insects and express higher levels of toxin than those expressing the longer Bt:NPT23 protein (Table 2). The Bt:NPTII fusion proteins detected in leaf extracts of the transformed plants had the expected size, as determined in Western blots. NPTII enzyme activity was exclusively associated with these fusion proteins as determined by *in situ* NPTII assays. Production of incomplete proteins or degradation in the plant cells was not observed. Thus, transformed tobacco plants produced fully functional fusion proteins of the size of the intact gene product.

The steady-state *Bacillus* messenger RNA levels in the transgenic plants were low and could not be reliably detected in Northern blot analysis. Therefore, they were quantified using ribonuclease protection experiments[18]. A probe containing the 5′ fragment of the *bt2* coding sequence up to nucleotide position 186 was synthesized using the SP6 transcription system and annealed to total RNA from the leaves of transgenic plants. After ribonuclease digestion, the protected fragments were run on a denaturing polyacrylamide gel. The results showed that RNA levels in the leaves of the transformed plants were correlated with *B. thuringiensis* protein levels (data not shown). The *B. thuringiensis* mRNA in N28-16, the plant that produces the highest level of protein, corresponds to ~0.0001% of the poly(A)$^+$ mRNA. The *Bacillus* protein detected in this plant represents 0.02% of the total soluble protein, or 3 μg of this protein per gram fresh leaf tissue. A fivefold lower level, as present in plant N28-34, was sufficient to induce 100% killing in a 6-day insect assay (Table 2). For comparison, the expression

levels of the intact *neo* gene also driven by the TR 2′ promoter, varied between 10 and 40 μg of NPTII per gram leaf tissue (0.07–0.27% of total protein). This difference in amount of protein is consistent with the lower kanamycin resistance levels of plants transformed with the *bt*: *neo* fusions compared to those transformed with the intact *neo* gene (Table 1).

Inheritance of the protection

Eighty-five transgenic tobacco plants, transformed with the four types of chimaeric *B. thuringiensis* genes and expressing various levels of active insect toxin, were grown in the greenhouse. All grew normally and were indistinguishable from controls in morphology and vigour of growth. We analysed the inheritance of the kanamycin resistance in eleven plants expressing *bt*: *neo* genes. Most of these plants contained one (N21-23, N21-50, N28-31, N28-34, N28-21) or two (N21-35, N28-19, N28-24, N28-32) kanamycin resistance loci, as confirmed by Southern blot analysis. Interestingly, some plants that were recognized as producing a large amount of *Bacillus* protein, such as N21-11 and N28-16, generated exclusively kanamycin-resistant F_1 seedlings. DNA analysis showed the presence of at least five copies of the T-DNA in these plants.

F_1 progeny from some transgenic plants were assayed for the expression of insecticidal activity. Insect toxicity was correlated with the kanamycin resistance marker in the F_1 progeny of plants N21-23, N21-50 and N28-34. Insecticidal activity was similar to that observed in the parental plants. Approximately 15 F_1 progeny of N21-11 and N28-16 were analysed. They all induced 100% killing of *M. sexta* larvae in the standard 6-day assay. Toxin levels in the F_1 progeny of N21-11 varied between 20 and 50 ng per mg of total protein, comparable to the 30 ng per mg in the parental plant.

Discussion

Four chimaeric genes containing modified *Bacillus* toxin genes under the control of the 2′ promoter of the *Agrobacterium* TR DNA, have been transferred into tobacco plants. All contain the toxic core of the Bt2 protein; *bt2* encodes the complete M_r 130K protoxin, *bt884* is a 5′ fragment of *bt2* up to codon 610. *Bt*: *neo23* and *bt*: *neo860* encode fusion proteins which are relatively stable, both in bacteria and plants, and which retain full insect toxicity and NPTII enzyme activity.

Insecticidal levels of toxin were produced when truncated *Bacillus* genes or fusion constructs were expressed in transgenic plants. Mortality rates among *M. sexta* larvae feeding on transgenic leaf material depended on the amount of toxic polypeptide produced. Typically, greenhouse grown plants producing more than 0.004% of their protein as the toxin produced 100% mortality in 6-day feeding assays. Some of the plants we have selected, contain toxin at three to five times this level (N21-11 and N28-16). In greenhouse tests, these plants were well protected from leaf damage caused by insects. Quantification also

Table 2 Insecticidal activity and *B. thuringiensis* protein content of transformed plants

Toxic protein	Plant	*Bacillus* protein detected (ng per mg total protein)	Insecticidal effect (% mortality) Day 3	Day 4	Day 6	Weight reduction in surviving larvae (%)
Bt: NPT23	N21-11	33 (132)	90	100	100	—
	N21-35	6.9 (71)	0	40	90	57
	N21-17	2.6	0	15	75	71
	N21-18	5.7	25	50	90	80
	N21-32	2.5	5	40	50	39
	N21-41	4.3	0	0	15	34
	N21-43	4.7	10	40	80	66
Bt: NPT860	N28-16	42 (190)	100	100	100	—
	N28-34	6.9 (42)	70	85	100	—
	N28-6	13	80	90	100	—
	N28-15	10	75	95	100	—
	N28-19	6.2	35	65	100	—
	N28-21	7.0	45	75	100	—
	N28-24	12	60	90	100	—
	N28-31	6.3	55	80	100	—
	N28-32	14	85	90	100	—
Bt2	N21-105	1.3 (5.5)	0	5	15	7
	N21-225	1.2 (2.1)	5	10	20	17
	N21-201	<1	0	5	10	23
	N21-236	1.3	0	0	0	0
	N21-238	<1	0	0	0	0
	N21-249	1.8	0	10	10	10
Bt884	N28-212	30 (125)	100	100	100	—
	N28-220	11 (40)	60	95	100	—
	N28-219	11	55	65	95	85

Values in parentheses refer to amounts detected in greenhouse grown plants. Extracts were prepared from leaves of propagated *in vitro* plants or from leaves of plants grown in a greenhouse that had between six and eight fully expanded leaves. Leaf tissue was ground up and subsequently sonicated (10 s at 50 W) in extraction buffer (Na_2CO_3, 50 mM at *p*H10; dithiothreitol (DTT), 5 mM; leupeptin, 1 mg ml^{-1}; Triton X-100, 0.05%; EDTA 50 mM; phenylmethylsulphonyl fluoride (PMSF), 0.19 mg ml^{-1}). The extract was cleared by centrifugation and *B. thuringiensis* polypeptides in the supernatant were quantified using an indirect enzyme-linked immunosorbent assay[22] (ELISA). Polyvinyl microtitre plates were coated with a goat antibody against *B. thuringiensis* crystal protein. Plant extract dilutions were incubated at 4 °C for 2 h in the coated wells. After rinsing, bound antigen was reacted with a mixture of four distinct monoclonal antibodies against Bt2 and subsequently with an alkaline phosphatase conjugated goat anti-mouse immunoglobulin antibody. The bound enzyme conjugate was detected by adding *p*-nitrophenyl phosphate as a substrate, and relative quantities were determined by measuring absorbance values at 405 nm. The monoclonal antibodies used specify antigenic epitopes located between amino-acid positions 29 and 222 in the NH_2-terminal region of the Bt2 protein (H. Vanderbruggen *et al.*, in preparation). To quantify the *Bacillus* protein levels in transgenic plants, ELISA binding curves of leaf extracts were compared to standard binding curves, obtained by diluting known quantities of the purified homologous protein in control extracts from non-transformed SR1 tobacco plants. The detection limit of the test for purified, solubilized Bt2, was 0.1–1.0 ng ml^{-1}. Toxin levels in plant leaves are expressed as ng toxin per mg of total soluble protein.

showed that the toxin expressed in plants has the same specific activity as in a bacterial host.

No significant insecticidal activity could be obtained using the intact *bt2* coding sequence, despite the fact that the same promoter was used to direct its expression. Intact Bt2 protein and RNA amounts in the transgenic plant leaves were 10–50 times lower than those for the truncated *B. thuringiensis* polypeptide or the fusion proteins. Expression levels were not significantly influenced by fusing the *neo* gene to the *bt2* sequence, but rather by the length of the *bt2* fragment. Why the complete *bt2* gene is not expressed at an equally high level in plant cells, is not known. Several parameters, such as differential RNA stability and translation efficiency might be important.

We observed in transgenic plants containing the *bt*: *neo* fusion constructs a correlation between insecticidal activity and resistance to kanamycin. Three-quarters of all plants resistant to 1,000 $\mu g\ ml^{-1}$ kanamycin induced 75–100% insect mortality. Such fusion genes can be used to select efficiently for transformed plants expressing strong insecticidal activity through direct selection for high kanamycin resistance.

Our experiments illustrate the feasibility of engineering plants that defend themselves against lepidopteran insects which are sensitive to the *B. thuringiensis berliner* insect toxin. However some species, such as *Heliothis* and *Spodoptera* which belong to the *Noctuidae*, an important group of pest insects, are less sensitive to common strains of *B. thuringiensis*, including berliner 1715 (ref. 19). To protect plants fully against these insects, higher levels of expression will be required. This might be achieved using chimaeric *Bacillus* genes containing stronger plant-specific promoters. The 35S promoter of cauliflower mosaic virus[20], for example directs a 10–50-fold higher expression than the regular T-DNA promoters in plants. Alternatively, it may be possible to construct chimaeric toxin genes with higher specific activity against important target insects. Transfer of different chimaeric genes into a variety of crops may provide a new and environmentally safer method of controlling destructive insect pests.

We thank Hilde Van De Wiele, Anne De Sonville, Veronique Gosselé, Jan Dockx, Annemie Van Houtven and Carine Matthijs for their contribution to the experiments. We also thank Leonor Fernandez for her contribution to the initial experiments, Danny Degheele for advice on insect tests, Karin Tenning for preparation of the manuscript, and Karel Spruyt and Stefaan Van Gijsegem for the figures.

Received 3 March; accepted 31 April 1987.

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EXHIBIT D

Sequence Alignment between Cry2Ae

and the Cry1A proteins of Cry1Aa1 (Barton); Cry1Ab5 (Vaeck); and Cry1Ab10 (Fischhoff)

Alignment: Global Protein alignment against reference molecule
Parameters: Scoring matrix: BLOSUM 62

Reference molecule: Cry2Ae1, Region 1 to 632
Number of sequences to align: 4
Total length of aligned sequences with gaps: 1488 aas
Settings: Similarity significance value cutoff: >= 60%

Summary of Percent Matches:

Ref:	Cry2Ae1	1 to	632	(632 aa)	--
2:	Cry1Aa1	1 to	1176	(1176 aa)	14%
3:	Cry1Ab5	1 to	1155	(1155 aa)	15%
4:	Cry1Ab10	1 to	1156	(1156 aa)	15%

```
Cry2Ae1          1 mnnvlnngrtticdaynvvahdpfsfehksldtirkewmewkrtdhslyvapivgtvssf
Cry1Aa1          1 ---mdnnpninecipyncls-------npevevlggerietgytpidisl-----sltqf
Cry1Ab5          1 ---mdnnpninecipyncls-------npevevlggerietgytpidisl-----sltqf
Cry1Ab10         1 ---mdnnpninecipyncls-------npevevlggerietgytpidisl-----sltqf

Cry2Ae1         61 llkk----vgsligkrilselwglifpsgstnlmqdilreteqflnqrlntdtlarvnae
Cry1Aa1         46 llsefvpgagfvlg--lvdiiwgifgpsqwdafpvqi----eqlinqrieefarnqaisr
Cry1Ab5         46 llsefvpgagfvlg--lvdiiwgifgps----qwdaflvqieqlinqrieefarnqaisr
Cry1Ab10        46 llsefvpgagfvlg--lvdiiwgifgps----qwdaflvqieqlinqrieefarnqaisr

Cry2Ae1        117 leglqanirefnqqvdnf-lnptqnpvplsitssvntmqqlflnrlpqfrvqgyqllllp
Cry1Aa1        100 leglsnlyqiyaesfreweadptnpalreemriqfndmnsalttaipllavqnyqvplls
Cry1Ab5        100 leglsnlyqiyaesfreweadptnpalreemriqfndmnsalttaiplfavqnyqvplls
Cry1Ab10       100 leglsnlyqiyaesfreweadptnpalreemriqfndmnsalttaiplfavqnyqvplls

Cry2Ae1        176 lfaqaanmhlsfirdvvlnadewgisaatlrtyqnylknytteysnycintyqt------
Cry1Aa1        160 vyvqaanlhlsvlrdvsvfgqrwgfdaatinsryndltrlignytdyavrwyntglervw
Cry1Ab5        160 vyvqaanlhlsvlrdvsvfgqrwgfdaatinsryndltrlignytdhavrwynt------
Cry1Ab10       160 vyvqaanlhlsvlrdvsvfgqrwgfdaatinsryndltrlignytdhavrwynt------

Cry2Ae1        230 ------------------------------------------------------------a
Cry1Aa1        220 gpdsrdwvrynqfrreltltvldivalfsnydsrrypirtvsqltreiytnpvlenfdgs
Cry1Ab5        214 ------------------------------------------------------------g
Cry1Ab10       214 ------------------------------------------------------------g

Cry2Ae1        231 fr---glntrlhdml---efrtym-fln-vfeyvsiwslfk---yqs-------------
Cry1Aa1        280 fr---gmaqrieqni---rqphlmdilnsitiytdvhrgfn---ywsghqitaspvgfsg
Cry1Ab5        215 lervwgpdsr--dwirynqfrrel-tlt-vldivslfpnydsrtypi-------------
Cry1Ab10       215 lervwgpdsr--dwirynqfrrel-tlt-vldivslfpnydsrtypi-------------

Cry2Ae1        267 -----------------llvs---------------------------------------
Cry1Aa1        331 pefafplfgnagnaappvlvsl---------------------------------------
Cry1Ab5        258 -----------------rtvsqltreiytnpvlenfdgsfrgsaqgiegsirsphlmdil
Cry1Ab10       258 -----------------rtvsqltreiytnpvlenfdgsfrgsaqgiegsirsphlmdil

Cry2Ae1        271 ----------------sganlyas-----------------------------gsgpqq
Cry1Aa1        353 ----------------tglgifrtlssplyrriil-------------------gsgpnn
Cry1Ab5        301 nsitiytdahrgeyywsghqimaspvgfsgpeftfplygtmgnaapqqrivaqlgqgvyr
Cry1Ab10       301 nsitiytdahrgeyywsghqimaspvgfsgpeftfplygtmgnaapqqrivaqlgqgvyr

Cry2Ae1        285 tqsftsqdwpf----------------------------ly-------slfqv---n---
Cry1Aa1        378 qelfvldgtef----------------------------sf-------asltt---nlps
Cry1Ab5        361 tlsstlyrrpfniginnqqlsvldgtefaygtssnlpsavyrksgtvdsldeippqn---
Cry1Ab10       361 tlsstlyrrpfniginnqqlsvldgtefaygtssnlpsavyrksgtvdsldeippqn---

Cry2Ae1        304 ------------------------------------------sny--------------
Cry1Aa1        400 tiyrqrgtvdsldvippqdnsvppragfshrlshvtmlsqaagavy--------------
Cry1Ab5        418 ----------------------------------------nnvpprqgfshrlshvs
Cry1Ab10       418 ----------------------------------------nnvpprqgfshrlshvs
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Cry2Ae1          307 -vlng--fsgarlt------------qtfpnigglpgtttthallaarvnysggvs---
Cry1Aa1          446 -tlraptfswqhrs------------aefnni--ipssqitqipltkstnlgsgtsvvk
Cry1Ab5          435 mfrsg--fsnssvsiirapmfswihrsaefnni--ipssqitqipltkstnlgsgtsvvk
Cry1Ab10         435 mfrsg--fsnssvsiirapmfswihrsaefnni--ipssqitqipltkstnlgsgtsvvk

Cry2Ae1          348 -----sgdi-----------------------------gavfnqnfscstflpplltpf
Cry1Aa1          490 gpgftggdilrrtspgqistlrvnitaplsqryrvririyasttnlqfhtsidgrpinqgn
Cry1Ab5          491 gpgftggdilrrtspgqistlrvnitaplsqryrvririyasttnlqfhtsidgrpinqgn
Cry1Ab10         491 gpgftggdilrrtspgqistlrvnitaplsqryrvririyasttnlqfhtsidgrpinqgn

Cry2Ae1          373 vrswldsgsd--rggvntv---tnwqtesfestlglrcgaftargnsnyfp---------
Cry1Aa1          550 fsatmssgsnlqsgsfrtvgfttpfnfsngssvftlsahvfns-gnevyidriefvpaev
Cry1Ab5          551 fsatmssgsnlqsgsfrtvgfttpfnfsngssvftlsahvfns-gnevyidriefvpaev
Cry1Ab10         551 fsatmssgsnlqsgsfrtvgfttpfnfsngssvftlsahvfns-gnevyidriefvpaev

Cry2Ae1          419 --------------------------------dyfirnisgvplvvrne-----------
Cry1Aa1          609 tfeaeydleraqkavnelftssnqiglktdvtdyhidqvsnlveclsdefcldekqelse
Cry1Ab5          610 tfeaeydleraqkavnelftssnqiglktdvtdyhidqvsnlveclsdefcl--------
Cry1Ab10         610 tfeaeydleraqkavnelftssnqiglktdvtdyhidqvsnlveclsdefcl--------

Cry2Ae1          436 ---------dlrrplh-----------------------------------------------
Cry1Aa1          669 kvkhakrlsdernllqdpnfrginrqldrgwrgstditiqggddvfkenyvtllgtfdec
Cry1Ab5          662 ---------dekkelsekvkhakrlsdernllqdpnfrginrqldrgwrgstditiqggd
Cry1Ab10         662 ---------dekkelsekvkhakrlsdernllqdpnfrginrqldrgwrgstditiqggd

Cry2Ae1          443 ----------------------yneirniespsgtpgglraymvsv---------hnrkn
Cry1Aa1          729 yptyl-----------------yqkidesklkaytryqlrgyiedsqdleiylirynakh
Cry1Ab5          713 dvfkenyvtllgtfdecyltylyqkidesklkaytryqlrgyiedsqdleiylirynakh
Cry1Ab10         713 dvfkenyvtllgtfdecyptylyqkidesklkaytryqlrgyiedsqdleiylirynakh

Cry2Ae1          472 niyavhengtmihlapedytg--------ftis-p------------------------
Cry1Aa1          772 etvnvpgtgslwplsaqspig--------kcge-pnrcaphlewnpdldcscrdgekcah
Cry1Ab5          773 etvnvpgtgslwrlsapspigkcahhshhfsldid-------------------------
Cry1Ab10         773 etvnvpgtgslwplsapspigkcahhshhfsldid-------------------------

Cry2Ae1          498 ----------ihatqvnnqtrtfi---------sekfgnq-----------------gd
Cry1Aa1          823 hshhfsldidvgctdlnedlgvwv---------ifkiktqdgharlgnlefleekplvge
Cry1Ab5          808 ----------vgctdlnedlgvwv---------ifkiktqdgharlgnlefleekplvge
Cry1Ab10         808 ----------vgctdlnedlgvwvifkiktqdgherlgnleflegraplv--------ge

Cry2Ae1          521 sl----rfeqsnttarytlrgngn------------------------synlyl------
Cry1Aa1          874 alarvkraekkwrdkreklewetnivykeakesvdalfvnsqydqlqadtniamihaadk
Cry1Ab5          849 alarvkraekkwrdkreklewetnivykeakesvdalfvnsqydrlqadtniamihaadk
Cry1Ab10         850 alarvkraekkwrdkreklewetnivykeakesvdalfvnsqydrlqadtniamihaadk

Cry2Ae1          547 rvsslg------------nstirvtingrvytasnvntttn--ndgvndngarfldinm
Cry1Aa1          934 rvhsireaylpelsvipgvnaaifeelegriftafslydarnviknqdfnnglscwnvk-
Cry1Ab5          909 rvhsireaylpelsvipgvnaaifeelegriftafslydarnviknqdfnnglscwnvk-
Cry1Ab10         910 rvhsireaylpelsvipgvnaaifeelegriftafslydarnviknqdfnnglscwnvk-

Cry2Ae1          592 gnvvasdntn------------vp---------------------------------ld
Cry1Aa1          993 ghvdveeqnnqrsvlvlpeweaevs-------------------------------qe
Cry1Ab5          968 ghvdveeqnnhrsvlv-------vpeweaevsqevrvcpgrgyilrvtaykegygegcvt
Cry1Ab10         969 ghvdveeqnnhrsvlv-------vpeweaevsqevrvcpgrgyilrvtaykegygegcvt

Cry2Ae1          606 invtfnsg----------------tqfelmn----im----------------------
Cry1Aa1         1020 vrvcpgrgyilrvtaykegygegcvtiheienntdelk---------------------
Cry1Ab5         1021 iheiennt----------------delkfsn----cveeevypnntvtcndytatqeey
Cry1Ab10        1022 iheiennt----------------delkfsn----cveeevypnntvtcndytatqeey

Cry2Ae1          623 -------------------f-----------------------------------------
Cry1Aa1         1058 -------------------fsncveeeiypnntvtcndytvnqeeyggaytsrnrgynea
Cry1Ab5         1060 egtytsrnrgydgayesnss-----------------------------------------
Cry1Ab10        1061 egtytsrnrgydgayesnss-----------------------------------------
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Cry2Ae1          624 --vp----------------------------tnlppiy------------------
Cry1Aa1         1099 psvp----------------------------adyasvyeeksytdgrrenpcefnrg
Cry1Ab5         1080 --vpadyasayeekaytdgrrdnpcesnrgygdytplpagyvtkeleyfpetdkvwieig
Cry1Ab10        1081 --vpadyasayeekaytdgrrdnpcesnrgygdytplpagyvtkeleyfpetdkvwieig

Cry2Ae1              ----------------------------------------------
Cry1Aa1         1129 yrdytplpvgyvtkeleyfpetdkvwieigetegtfivdsvelllmee
Cry1Ab5         1138 etegtfivdsvelllmee--------------------------
Cry1Ab10        1139 etegtfivdsvelllmee--------------------------
```